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Veranus Alba Moore
1859-1931

Veranus Alva Moore was born at Houndsfield, Jefferson County, New York, April 13, 1859. His parents were Alva and Antoinette Eastman Moore. His father died when Veranus was thirteen years of age and the son worked on a farm to support himself and to assist his mother and the other children. In the course of his farm work he suffered a serious infection of the foot caused by stepping on a nail and the long treatment he received at Bellevue Hospital and elsewhere (in days when "Listerism" was a subject of merriment to the medical profession) awakened his interest in the obscure but fascinating problems of disease. In the intervals of hospital treatment he taught district school; and after studying at Mexico Academy in Oswego County he entered Cornell in the fall of 1883. He earned his way through college in spite of the fact that he still used crutches until his junior year. While at Ithaca, he specialized in pre-medical subjects and came under the inspiring influence of Burt G. Wilder and of James Law (who had studied with Lister at Edinburgh).

So faithful and successful was the work of Mr. Moore that the required studies for graduation had been completed in the early spring of his senior year (1887). At that time there came a request from Dr. D. E. Salmon, and Dr. Theobald Smith of the Bureau of Animal Industry in Washington, for an assistant. Mr. Moore was recommended for that position and was accepted, the university granting him leave of absence for the remainder of the school year. He returned and graduated with his class in June.

In the Bureau at that time were Daniel E. Salmon, by whose efforts the Bureau had been established, and Theobald Smith, whose epoch-making discovery of the intermediate host in the

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transmission of Texas fever to cattle paved the way for later discoveries concerning the rôle played by insects and their close allies in transmitting disease to human beings.

Moore's interest in medicine was further intensified in the atmosphere of scientific investigation at the Bureau. Fortunately for him, the Columbian Medical School (now George Washington University Medical School), held its lectures and laboratory work in the late afternoon and evening. After carrying on his scientific work all day in the Bureau, with unbounded energy he took up the study of medicine, completed the course and graduated in 1890 with the degree of M.D. He then was appointed demonstrator in the school, and from 1894 to 1896 served as professor of normal histology. When Dr. Theobald Smith went to Harvard in 1895, Dr. Moore served as chief of the division of pathology in the Bureau till 1896. At this time the New York State Veterinary College opened its doors, and Dr. Moore was called to the professorship of Pathology, Bacteriology and Meat Inspection. He built up an admirable department, and the services he rendered to the livestock owners of the state and the nation were widely recognized. He also did yeoman service in helping to free the veterinary profession and the community from opinions without foundation, and put in their place the sound knowledge which bacteriology and the new pathology had made available. To this end he published many papers (some 264), gave numerous addresses and prepared several books in his field. Among these are; Directions for Beginners in Bacteriology, The Pathology and Differential Diagnosis of Infectious Diseases of Animals, Bovine Tuberculosis and its Control.

On the establishment of the Cornell University Medical College, Dr. Moore gave instruction from 1898–1910 to the medical students in the Ithaca Division, and gave these young people a broad outlook upon comparative bacteriology and pathology.

In 1908, on the retirement of Dr. James Law at the age of seventy, Dr. Moore was chosen to succeed him as Dean of the Veterinary College, and continued in that office for twenty-one years. He brought to this important position the broad training which had come from his nine years at the Bureau of Animal

Industry in Washington with its national outlook, and an intimate knowledge of the actual problems confronting the stock owners of every county in New York State. In 1909 his experience was further increased by study and observation of the efforts toward tuberculosis control in cattle made in the several countries of Europe on a trip made in the interest of the New York State Department of Agriculture.

Dr. Moore brought with him to the deanship that precious quality of sympathy, which was his by nature and which had been greatly developed by his contact with the best minds in the country. He not only knew the needs of the stock owners of the state and country, but he knew the necessity of securing and retaining men of the highest grade for the teaching and research staff of the college.

During his twenty-one years administration as Dean, he had the satisfaction of seeing notable advances in every department of the college. When he became Dean the annual appropriation for maintenance was $30,000. So skillful was Dr. Moore in making known the widespread benefits arising from the work of the college, and so eloquent and persuasive was his presentation of the needs and the benefits to come, that, when he retired, the annual appropriation for maintenance had increased almost six fold, that is, to $178,955. The six buildings and equipment in 1908 had cost $150,000. The eight buildings and equipment added during his administration cost an additional $366,000. The staff of thirteen in 1908 had increased to thirty in 1929; there were 80 enrolled students in 1908, and 134 in 1929. Up to 1908 the college had graduated one hundred and fifty-three; to these were added 550, making a total alumni body of 703. Up to 1916 the course had been three years long, since then it has been four years.

As there was no place in the state where diagnoses could be made of such diseases as anthrax, blackleg, glanders, rabies, tuberculosis and other animal diseases, the Department of Bacteriology and Pathology established a laboratory for this purpose where specimens could be examined and information given. This diagnostic work has constantly increased in volume and importance.

It is almost self-evident that an institute like the veterinary college in a great university cannot limit its facilities to under-graduates. Provision must be made for graduate work, and opportunity offered to practitioners who wish to become ac-quainted with the latest knowledge and the latest methods.

Dr. Moore always had in mind the students and what was for their advantage, and after they became alumni he did not forget them. Every year just before Christmas he sent a letter to each, telling of the more important changes in the university as a whole, and then more particularly of the incidents in the past year at the college, the successes of the investigations by the staff and other matters which he thought might interest the alumni and make them feel that they were still a part of their *alma mater*. In 1909, he established the Conference of Veterinarians, a two-day session in which all matters of present vital interest to the profes-sion are taken up and considered by alumni and men eminent in the state and in the United States. There are also clinics in which the new things in surgery and medicine are demonstrated. Every year more and more alumni return to the conferences, and the non-alumni practitioners of the state are much in evidence.

Not alone in his official capacity as teacher and administrator did Dr. Moore meet the students and alumni on a friendly footing, but his home was open to them and they were sure of a friendly welcome by that beautiful household of father and mother, two sons and a daughter. What that friendly home influence has meant to the successive classes of the college, no one can estimate.

In its hour of need Dr. Moore did not forget his country, but served in the Surgeon General's office where he was assigned to the duty of helping to organize the veterinary corps of the United States Army.

Perhaps in no other way could be shown more clearly the honor and esteem in which Dr. Moore was held in the university community than by the statement that he was chosen by the University Faculty as one of its three representatives on the University Board of Trustees. This position he held from 1926 till his retirement in 1929.

On retiring from the Veterinary College in 1929 at the age of 70,

Dr. Moore had planned to devote his remaining years to a quiet life of research, and especially to the preparation of a history of Veterinary Medicine in America, but circumstances determined otherwise. He had been on the Ithaca Board of Health for twelve years, and had served for twelve years on the Board of Education. He had been a trustee of the Ithaca hospital since 1918, and a member of the medical staff from its foundation. He had urged and helped to bring into being the county laboratory, the contagious wing of the hospital and the nurses' home. It was perfectly natural therefore for the trustees of the hospital to turn to Dr. Moore, who to them seemed free, and ask him to assume the duties of superintendent, and, with his tried skill in institutional management, to straighten out the tangled finances, stop the growing deficit, and bring about genuine team work among all connected with the hospital.

Dr. Moore put aside his plans for freedom from administrative cares because there seemed to be here a place where he could be of real service. He brought to this position very definite knowledge of the best practice in hospital management at the present day, and knew all too well its defects in earlier years. He brought also the precious faith in human nature which he believed could be counted on when rightly appealed to. One of his first efforts when he assumed the position in the autumn of 1929 was to try to make all connected with the hospital from the visiting physicians to the lowest employee see what to him was so clear: "that in a hospital, the patient is always first."

As a patient in Bellevue, Dr. Moore had seen the beginnings of the first training school for nurses in the country. He naturally therefore had a deep interest in the nurses' training school in the Ithaca hospital, and had far-seeing plans for improving it, and for making it one of the leading schools in the country by a combination with the College of Home Economics in Cornell University.

All of his constructive work, and plans were making notable progress when, alas, he himself came to need the ministrations of his own hospital. There on the morning of February 11, 1931 he passed from sleep in life to the dreamless sleep of death.

SIMON HENRY GAGE.

ULTRAFILTRATION STUDIES ON THE VIRUS OF POLIOMYELITIS[1]

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Ultrafiltration study of the ultramicroscopic viruses provides a direct method for determining the approximate size of these agents. A graded series of collodion filters of progressively smaller permeability can be prepared according to the general method developed by Bechhold (1907a, 1907b) and applied to biological studies by Hitchcock (1925, 1926), Elford (1929), Krueger and Ritter (1930), and others.

Under appropriate conditions the virus suspensions can be fractionated into a series of suspensions containing more and more finely dispersed particles, the series of membranes acting in some respects as a graded sieve. Results obtained by this method, therefore, give only an approximation as to the maximum size of the active principle.

In this work, before carrying on ultrafiltration studies with the virus of poliomyelitis, a comparison was made between membranes prepared according to the different methods described in the literature. On the basis of these preliminary studies the following procedure was adopted, for the reason that by this method membranes can be reproduced with an accuracy of $\pm$ 5 per cent. Whatman No. 1 filter papers are immersed in the acetic acid solution of collodion after a preliminary bath in glacial acetic acid to displace air. To insure thorough impregnation the papers are soaked in the collodion solution for at least fifteen hours.

They are then slowly and uniformly lifted in a vertical position from the bath, the excess collodion being removed by the combined effects of drainage and surface tension. The impregnated papers are then plunged into sterile distilled water to gel the collodion.

To obtain a wide range in pore size, solutions containing from 0.5 to 8.0 per cent nitrocellulose (Eastman Kodak Company) by weight in glacial acetic acid may be used. To obtain reproducible results these solutions must be allowed to stand at least one month before they are employed or be "aged" by heating for several hours at 90 to 95°C. After this time the viscosity of the solutions remains constant.

The membranes must be repeatedly washed with sterile distilled water to remove the acetic acid. The presence of acid within the pores may cause proteins to precipitate during filtration and therefore it is advisable to wash the membranes a number of times with sterile 0.1 per cent Na_2CO_3 solution before filtering the virus suspensions.

In ultrafiltration studies it is assumed that the membrane acts as a sieve, composed of a large number of extremely fine capillaries at right angles to the filtering surface. By determining the rate of flow of water under standard conditions through the membrane it is possible to calculate the pore size by means of Poiseuille's law which is expressed in the following equation:

$$d = 4\,l\,\sqrt{\frac{2\,Q\,\eta}{PV}}$$

where d = diameter of pores
$\quad\quad\; l$ = thickness of membrane
$\quad\quad Q$ = amount of flow in grams per second
$\quad\quad \eta$ = viscosity of water
$\quad\quad P$ = filtration pressure in dynes
$\quad\quad V$ = volumes of pores

Values for pore size determined in this manner are greater than those obtained by filtration of colloidal particles of known size. This difference increases as the pore size of the membranes is decreased.

The values obtained from rate of water flow represent a reliable criterion of the reproducibility of membranes prepared by any method and probably closely approximate the average pore size. In the calibration of the membranes by this method it is necessary to support the ultrafilter on a finely perforated metallic disc to prevent undue strain on the collodion membrane with resulting collapse of the pores.

In a series of experiments in which the membranes were supported on discs having a circular opening 1, 2, and 3.0 cm. in diameter, respectively, the rate of flow increased approximately in the ratio of $1:3:5$, rather than in the ratio of filtration surface $(1:4:9)$. Somewhat closer agreement was obtained with less permeable membranes.

The support finally adopted for the membranes has 24 openings, each 0.24 cm. in diameter. Only a small difference is noted between the rate of flow per unit area through this disc and the rate of flow through the disc provided with a single opening of 1.0 cm. in diameter. However, higher filtration pressures can be used with the perforated plate support and this with less strain on the membranes than is the case when one uses a plate with an equivalent single opening.

The rate of flow through the different membranes was determined for positive pressures ranging from 2.5 to 15.0 cm. Hg. A straight line relationship between rate of flow of water and filtration pressure was obtained. It is advisable to apply a pressure of 15 cm. Hg. for a period of several minutes before starting a test in order to obtain good contact between the membrane and the supporting disc. This tends to prevent leakage between the surfaces of the membrane and disc. Thereafter the rate of flow of water through the membranes can be more accurately determined for a given range of pressures.

Some investigators claim that certain viruses pass through a filter more readily when suspended in broth. This may be true for porcelain filters, but studies on the rate of flow of broth through collodion membranes indicate that the proteins in the broth markedly clog the pores of the membrane.

By the use of a series of colloidal sols it is possible to determine

what size particles are retained by a given membrane. If the same technique is employed for the filtration of the ultramicroscopic viruses a comparison can readily be made between the particle size of the colloids and the virus, provided the virus can be obtained in a sufficiently pure state. The values obtained in this manner probably give a more accurate estimate of the effective pore size in any ultrafiltration studies on colloidally dispersed systems.

The pore radius for the denser membranes as determined by rate of flow studies is on the average 60 to 70 $\mu\mu$ greater than the values obtained by filtration of colloids. This discrepancy is probably due to interfacial tension effects; negative adsorption in which water is preferentially adsorbed in the pores as an immobile layer; or to blocking of the pores, either as the result of positive adsorption of the colloidal particles or by mechanical means.

In the filtration of virus suspensions the rate of flow decreases rapidly, due to adsorption or the deposition of a protein layer in the pores as well as on the surface of the membrane. This can be prevented to some extent by constantly stirring the suspension during ultrafiltration. In many cases, especially when organic colloids are used, filterability is increased if the surface tension of the dispersion medium is depressed by means of sodium ricinoleate or other surface tension depressants.

The conception of a mechanical sieve action for the filtration of colloids through these membranes is in itself untenable but we are probably justified in comparing the results obtained in ultrafiltration of the viruses with those of known colloidal suspensions. In studies such as these one must bear in mind the possibility that the virus may actually pass through denser membranes, but in concentrations which are not sufficient to yield a positive biological test of its presence. This greatly reduces the delicacy of any ultrafiltration procedure employed with the idea of determining the actual particle size of a given virus. For this reason determinations such as these can only indicate that the particle size of the virus is actually less than the diameter of the pores of the membrane through which it is found to pass. With increase

in purity of the virus suspension filterability is appreciably increased and progressively smaller values are obtained for the size of these agents.   This is clearly shown if one reviews the literature on the determination of the size of bacteriophage by ultrafiltration methods.

Krueger and Schultz (1929) in ultrafiltration studies on the virus of poliomyelitis found that this agent possesses a magnitude not greater than 300 $\mu\mu$.

In the studies which we are now reporting the lipoids and some of the protein in the virus suspensions were removed by ether extraction using the following method: A weighed portion of the cord and medulla from a poliomyelitis monkey which had succumbed to complete flaccid paralysis is finely ground with pyrex glass in a mechanically driven mortar.   Enough phosphate buffer solution (pH 7.4) is added to facilitate proper grinding.   After the desired degree of dispersion is obtained, the material is suspended in a measured volume of the phosphate buffer and made up to a 5 per cent suspension.   The coarser particles are allowed to settle in the ice box for about one hour.   The supernatant fluid is then centrifuged at about 1250 r.p.m. for fifteen minutes. This clarified liquid is placed in a glass stoppered bottle with an equal volume of ether and mechanically shaken for one-half hour.   On standing in the ice box the mixture separates into 3 layers consisting of (1) an upper layer of an ether solution of lipoidal matter, (2) a middle or proteinaceous-lipoidal layer and (3) a bottom aqueous layer.   If the extraction is properly carried out the aqueous fraction is almost water clear.   Repeated takes have been obtained in monkeys following intracerebral injection of this fraction without a single failure.   On the other hand inoculation of the ether fraction, with only a few exceptions, has never produced poliomyelitis in monkeys.

If the aqueous layer is slightly turbid it can be clarified by centrifuging at 1600 to 1800 r.p.m., and the supernatant liquid filtered through a sterile Whatman No. 1 filter paper.   The ether is removed in vacuo and the virus suspension is then ready for use.   These procedures must be carried out under sterile conditions.

With this semi-purified suspension of the virus we have been able to obtain repeated "takes" in monkeys with filtrates through 3 per cent membranes. Filtration through these membranes was either facilitated by mechanical stirring or by the addition of sodium ricinoleate, which served to decrease the surface tension of the virus suspension. It was noted in these studies that at least 40 to 50 cc. should be filtered. Since the concentration of virus in the first portion of the filtrate is materially decreased by adsorption, the first 10 to 20 cc. should be discarded.

Our results thus far indicate that the magnitude of this virus is below 110 $\mu\mu$ when based on permeability of the membranes to water, or 50 $\mu\mu$ when based on permeability to colloidal particles. If the permeability of the membranes to water is tested before and after 10 cc. of the virus suspension have been filtered a marked decrease in permeability is noted. This therefore indicates that if only the last portion of the filtrate is tested we are justified in assuming that the magnitude of the virus is somewhere below 50 $\mu\mu$ in diameter. The actual size of the virus may lie considerably below this value, since at this stage it is impossible to eliminate the possibilities of the virus being adsorbed to proteins or other colloidal aggregates. Further purification and ultrafiltration studies are in progress.

These filtrations were carried out under aseptic conditions and neither aerobic nor anaerobic cultures from the filtrates revealed the presence of streptococci or other microörganisms. Nevertheless, typical symptoms of experimental poliomyelitis were produced in monkeys following the injection of these filtrates.

SUMMARY

A simple procedure is described for the preparation of acetic-collodion membranes for ultrafiltration studies, and the principles of ultrafiltration are discussed. Results in our hands indicate that the magnitude of the virus of poliomyelitis lies below 50 $\mu\mu$ in diameter.

REFERENCES

BECHHOLD, H. 1907 Biochem. Z., **6**, 379.
BECHHOLD, H. 1907 Z. Phys. Chem., **60**, 257.
BECHHOLD, H. 1908 Z. Phys. Chem., **64**, 328.
ELFORD, W. J. 1929 Brit. Jour. Exp. Path., **10**, 126.
HITCHCOCK, D. I. 1925 Jour. Gen. Physiol., **8**, 61.
HITCHCOCK, D. I. 1926 Jour. Gen. Physiol., **9**, 755.
KRUEGER, A. P., AND RITTER, R. C. 1930 Jour. Gen. Physiol., **13**, 409.
KRUEGER, A. P., AND SCHULTZ, E. W. 1929 Proc. Soc. Exp. Biol. and Med., **26**, 600.

THE BACTERIOLOGY OF PULP SLIME

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The occurrence of slime in various stages of the manufacture of pulp for paper stock constitutes a problem of increasing magnitude. The effects of slime growths within pulp mills are well understood. Holes, characterized by a burnished and shiny margin are produced within the finished paper. In addition, accumulation of this thinly gummy material dispersed through the stock in finer particles injures the texture of the paper and is said even to interfere with proper sizing.

The purpose of this study then was to determine if possible the cause of paper slime and also to ascertain certain facts dealing with its derivation.

There have been many assumptions concerning the nature of the cause of this difficulty. Most of these guesses have been based upon the logical idea that some microörganism is an etiological factor but these hypotheses have not been founded upon experimental evidence. Algae, fungi including yeasts, and bacteria have all been incriminated.

If direct microscopical stains be made of this slime, a variety of forms will be found to be present, for the picture will include in addition to many finely divided but amorphous particles, a variety of bacteria. These are largely Gram-negative although some of them are Gram-positive. Those which are negative are usually three or four times as long as they are thick and are pleomorphic. A few mycelial threads of fungi will be noted but these are likely to be irregular in tinctorial reaction and may appear to be degenerating. An occasional yeast cell is evident. No algae are present. By process of exclusion then one is led by microscopic smear examination to seek the cause of slime produc-

tion within the pulp industry among the bacteria rather than among other groups of microörganisms.

If slime is caused by a bacterial organism, then one may speculate for a moment as to its structure and as to certain metabolic requirements of the form necessary in all probability to produce it. The consistency of this material reminds one of a bacterial zoogloea. Thus a capsule producing organism probably must be sought. For maximum building of capsule structure by bacteria, a supply of carbohydrate is essential. Zettnow (1918) has indicated stimulation to increased capsule formation by carbohydrate within the medium and Heidelberger and Avery (1923) have shown that the addition of glucose to a culture of pneumococcus increases capsule production many fold. Carbohydrate in such a form as to be usable for bacterial metabolism must therefore be available.

We are thus brought to the point of determining whether carbohydrate in form available for growth of zoogloea producing bacteria is present in white water. If present, it may be in solution within the water itself or it may be in close contact with the suspended cellulose or other colloidal material. Inasmuch as the various modes of treatment applied to cellulose are attended by a certain degree of hydrolysis, inversion of at least a small portion of the cellulose is to be expected. Indeed Lottermoser and Mathieson (1929) have shown a content of 1.4 per cent sugar in certain sulphite liquors and the largest fraction of this carbohydrate content exists as glucose. Moreover, we have shown that if one suspend bleached pulp in distilled water and then add either Fehling or Benedict solution, a definitely positive reaction for the presence of carbohydrate is obtained. Hexoses therefore are present here although they may be absent in unbleached pulp as proven by a similar test. Like tests made with white water at Berkeley have given negative results but the samples were at least five days old when received and thus carbohydrates had already doubtless been destroyed by microorganic fermentation. The above work, however, has proven that carbohydrate in relatively simple form is present within or in contact with the cellulose fiber and that it results in part at

least from hydrolytic activity of certain of the reagents used in processes of manufacture.

With these observations therefore as a background, a series of attempts have been made to isolate organisms from pulp slime which are to be characterized by copious formation of capsular substance.   For this purpose there have been utilized media rich in carbohydrate, particularly glucose.   The initial attempts were made by means of Sabouraud's agar compounded according to the following formula.

Water, distilled	1,000 cc
Peptone	10 grams
Glucose	40 grams
Dissolve these, then add·	
Agar	20 grams

This was used as poured plates.   In addition there were utilized the usual glucose beef agar containing 1 per cent of the carbohydrate and the ordinary plain beef agar with no carbohydrate added.

Streaks from pulp slime were made upon agar plates of these media and incubation followed.   For this purpose two temperatures have been used, 37°C. and 28°C. since the temperatures of the environment where slime is found suggests that these should be desirable.   Following two days at 37°C., or three days at 28°C., a variety of organisms will appear in colony formation. Among these, although not in pure culture, are certain colonies which if allowed to continue to grow will become 10 to 15 mm. in diameter and are characterized by a marked slimy consistency. The color will be slightly yellowish or grayish upon Sabouraud's media.   Their growth is so persistent that if proliferating near the edge of a Petri dish the colony may extend up the glass surface for 3 to 5 mm.   Mixed with these is a variety of organisms which are fairly constant over a series of such examinations for these additional forms constitute in part what appears to be a bacterial flora characteristic of white water.   In many instances these additional organisms far outnumber the zoogloea producers. Figure 1 is a photograph of two representative preparations made as described.

The cultural reactions of the organisms constituting the heavy mucilaginous colonies noted above may be determined following purification by plating or streaking upon Sabouraud's agar. They are as follows. Gram-negative, non-sporulating rod, 3 to 4 microns long by 1 micron thick, slightly motile, capsule positive by Gins' method. Gelatine is not liquefied. Indol production is negative. It grows luxuriantly upon Sabouraud's agar and also to less degree upon all the usual agar formulae utilized in routine laboratory practice. Growth upon media poor in carbohydrate however is not attended by formation of heavy capsu-
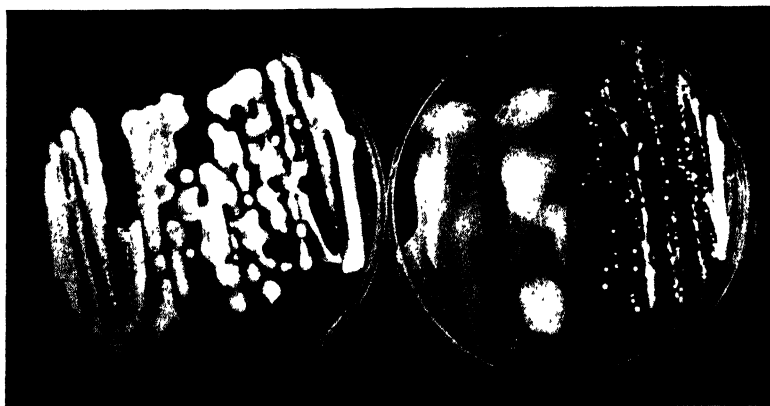
Fig. 1. Growth of Pulp Slime Organisms upon Sabouraud's Glucose Agar.
Primary Isolations
Organisms are absent from sample streaked at right

lar structure. Small amounts of H_2S are produced upon lead acetate medium. Nitrates are reduced to nitrites. The methyl-red reaction is negative while the Voges-Proskauer test is usually positive. Growth in plain beef broth is attended after some days by formation of a definite and heavy pellicle. Milk becomes acid in five days and some strains cause coagulation. Sugar reactions are the following: "Acid with gas from glucose, sucrose, salicin, maltose, raffinose, mannitol, levulose, galactose, and dextrin. This last is slow to react." Lactose is negative at first with some strains but becomes positive with attendant gas

production after two or three transfers. With other strains, both gas and acid are produced with freshly isolated organisms. Incubation temperatures of 30°C. or 37°C. are seemingly equally satisfactory. The organism grows best as an aerobe although it proliferates in deep beef agar without added sugar and under a vaseline seal. Under these conditions, gas is produced with disruption of the agar and it is likely that the carbohydrate content of the medium naturally present is now utilized.

This organism closely resembles *B. aerogenes* but it differs from it in certain details. Many freshly isolated cultures are inactive with lactose although they acquire ability to break down this carbohydrate easily following a few transfers. Slight yellowish pigmentation may appear with recently isolated strains. Capsule production upon carbohydrate-containing medium is very heavy. Nevertheless this form probably may be classified as *B. aerogenes*.

Inasmuch as it has become advisable to attempt to determine the presence of this organism in water which is to be used by pulp and paper mills, a method for enrichment for culture purposes has been elaborated. In its background, it is based upon the technique used for the sanitary examination of water. The procedure follows. Fifty cubic centimeter amounts of the water to be tested are first centrifugalized at high speed for one-half hour. The resulting sediment is then streaked out upon Sabouraud's medium, since in certain instances this process of concentration is sufficient to reveal the form, if present. It is more important however to place the sediment in glucose broth fermentation tubes with Andrade indicator. These preparations are made in duplicate sets. Of these sets, one is incubated at 37°C. while the other is placed in a chamber at 30°C. When acid and gas have been produced, the tube showing this reaction is now streaked out upon a poured plate of Sabouraud's agar which is then incubated. When, and if, the characteristic large thinly mucilaginous colonies develop, they may be picked and cultural reactions determined as outlined above. By this method the slime producing organism has been isolated from raw water taken from the vicinity of pulp mills upon a number of occasions.

The proof that this organism is capable of causing slime in

contact with cellulose is shown by the following test. Crude wood pulp is placed in flasks and to this is added Sabouraud's fluid which has the same formula as that used for the semi-solid medium except that the agar is omitted. Sufficient of the fluid is added to moisten the pulp thoroughly together with a little excess. It is of advantage to add a slight amount of powdered calcium carbonate in order to neutralize resulting acidity in part. The material is now sterilized by the intermittent method. The flask thus prepared is inoculated and then incubated at either 37°C. or 30°C. In four or five days the surface of the pulp shows increased reflection of light due to the accumulation of the capsular material and after two or three weeks, the whole mass has become sufficiently gummy so that the smaller particles stick together and pulp slime is thus produced artificially. For the final step of proof in a manner simulating the requirements of Koch's postulates, the original organism may be isolated from this preparation provided such isolation is made reasonably soon. This bacillus is killed after some days by accumulation of its own acid by-products and thus such preparations become sterile after a period of three or four weeks, particularly if buffer be absent. For this experiment, it is advisable to utilize freshly isolated cultures inasmuch as continued culture upon artificial media reduces somewhat ability to form zoogloea.

With the foregoing facts as basic observations it seems probable that pulp slime forms in the following manner. The capsule building bacteria proliferate at any convenient point of attachment within the white water channels and are transported there by the white water. The temperature of the white water is a limiting factor for growth and consequently sliminess cannot take place in a location which is too warm. These forms are not thermophilic. Twenty-eight to 40° is the optimum range although proliferation takes place more slowly at lower temperature. The carbohydrate content of the water due to hydrolysis of cellulose stimulates formation of bacterial gum. With formation of this mucilaginous coating, floating bits of cellulose are caught together with other finely granular detritus. This enmeshed mass contains the many bacterial organisms which constitute in part

the flora of white water. In addition mold spores and mycelium accumulate here. These latter forms have been described by Kress *et al.* (1925). Additional evidence of soluble carbohydrate at this spot is shown by the presence of a few yeast cells. The slime producing organisms therefore constitute but a small portion of the microörganic content of the pulp slime. When this zoogloea becomes over-cumbersome for its location, it floats off into the cellulose mass with consequent effects well recognized within the industry.

There is still an additional reason why the number of heavy capsule-producing organisms are found here in relatively small numbers. This lies in the fact that white water is but slightly buffered in spite of high colloidal content. These organisms are sensitive to their own acid by-products. Thus, they disappear rather readily from the mechanical structure which they have built up to the point that relatively few may appear within culture.

Questions may naturally be raised regarding the source of these bacteria which have been proven to be the cause of pulp slime. There have been two opinions regarding this matter. One group incriminates water as the cause of the difficulty while the other believes that the cause of the sliminess is to be found within the wood itself. In an endeavor to gain evidence as to this point, the following experiments have been carried out. As indicated previously it has been shown conclusively that this capsule-producing form is present in water. Such isolations have been made in many instances from raw waters in Washington and Oregon taken from rivers supplying pulp and paper mills. Water is at least one source of the difficulty therefore. It remains to determine whether these forms may not also be found in the wood itself. If a sound pulp log be sectioned under proper precautions so that portions from near the outside where water has penetrated may be compared bacteriologically with similar portions removed from near the center where water has not as yet penetrated, the flora are found to differ markedly. Slime-forming bacteria similar to these previously described are readily isolated from the water soaked portion but are absent in that part where water has

not arrived. The organisms moreover are to be found in the water of the pond in which the log has been floating. Thus it appears that the source of these forms is water and not wood. Methods of control therefore must concern water rather than wood.

CONCLUSIONS

The cause of pulp slime in paper and pulp mills is described together with methods for its isolation and recognition. Reasons for its growth are indicated.

The morphology and cultural reactions of an organism closely similar to *B. aerogenes* are outlined.

The source of the causative rod is water rather than wood and thus it is shown that methods of control should be directed towards water treatment.

REFERENCES

HEIDELBERGER, M., AND AVERY, O. T. 1923. The soluble specific substance of pneumococcus. Jour. Exp. Med., **38**, 73–79.

KRESS, O., *et al.* 1925. Control of Decay in Pulp and Pulp Wood. U. S. Dept. Agr. Bull. No. 1298.

LOTTERMOSER, A., AND METHIESEN, E. 1929. Uber Zucker in der Sulfitablauge. Tech. u. Chem. d. Papier u. Zellstoff-fabr., **26**, 37–42.

ZETTNOW. 1918. Klein Beiträge zur Morphologie der Bakterien. Zeit. f. Hyg. u. Infek., **85**, 17–32.

THE PRODUCTION OF YEAST-GROWTH STIMULANTS BY THE MOLDS

I. ASPERGILLUS NIGER, TRICHODERMA LIGNORUM, AND ASPERGILLUS CLAVATUS

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During the investigation of the growth of yeast on synthetic media it had been observed that occasionally a greatly enhanced growth was shown in media which had been accidentally contaminated by molds. This phenomenon was so striking as to warrant a systematic study of the production of yeast growth stimulants by several molds. The studies here reported include the molds *Aspergillus niger, Trichoderma lignorum,* and *Asp. clavatus*[1] and the yeast *Saccharomyces cerevisiae* (No. 4226 American Type Culture Collection). Further studies are in progress.

The subject of yeast growth stimulants known under the name of bios has been adequately reviewed up to 1925 by Tanner (1925) while later developments have been considered by Buchanan and Fulmer (1930).

Two media were used for the growth of the molds, one containing sucrose as a substrate and the other glycerol. The sucrose medium, medium C (Fulmer, Nelson and Sherwood (1921), Fulmer and Nelson, 1922) contained per 100 cc.: 0.188 gram NH_4Cl, 0.100 gram K_2HPO_4, and 10 grams sucrose. The glycerol medium used was a modified Czapek's medium as developed by Naylor, Weisbrodt-Smith, and Collins (1930) and contained per liter: 0.5 gram $MgSO_4$, 1 gram K_2HPO_4, 0.5 gram KCl, 0.01 gram $FeSO_4$, 5.3 grams NH_4Cl and 10 grams glycerol.

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[1] The molds were kindly furnished by Dr. J. C. Gilman of the Department of Botany.

The molds were allowed to grow for two weeks at about 20° in 500 cc. of medium in a 2-liter flask. The mats were removed and the medium passed through Berkfeld filters. The filtrates from the sucrose medium were used unaltered for the growth of the yeast except for the adjustment of the pH, while sucrose (100 grams per liter) was added to the glycerol medium filtrates. Growth of the yeast in these media was compared with that in similar media in which no mold had grown. The media were sterilized by filtration. This procedure was adopted in view of the findings of Fulmer and Huesselman (1927) that yeast growth

TABLE 1

Effect upon the growth of yeast of media which had supported the growth of Aspergillus niger

(The numbers in the columns multiplied by 250,000 give the number of yeast cells per cubic centimeter.)

	SUCROSE MEDIUM		GLYCEROL MEDIUM			
	I	II	III	IV	V	VI
Initial pH..........	6 9	6.9	6.9	6.9	7.5	7.5
	86	28	55	30	86	29
	97	25	54	32	71	29
	89	24	51	26	87	36
			49	29	76	42
			53	37		
			50	31		
Final pH...........	2 7	5.5	2 70	5.50	2.7	3.55

I, III, V = media had supported mold growth.
II, IV, VI = media had not supported mold growth.

stimulants may be formed during the sterilization of medium C under pressure. The media were inoculated with yeast to a count of one (250,000 cells per cubic centimeter) and incubated for forty-eight hours at 30° when the count was again determined.

In table 1 are given the results obtained by use of the media in which *Aspergillus niger* had been grown. It is at once evident that this mold produces yeast growth stimulants whether acting on sucrose or on glycerol.

Six liters of the filtrate from the glycerol medium which had supported the growth of the mold were evaporated to dryness at

70°. About 20 grams of a gray powder resulted. This material is not entirely soluble in water. A suspension of this residue was tested on the growth of yeast in medium C. The results are shown in table 2. These data indicate that the stimulating material is highly concentrated in this residue and is heat-stable.

TABLE 2

Effect upon the growth of yeast of dried residue from glycerol medium which had supported the growth of Aspergillus niger

$$\text{Count} = \frac{\text{yeast cells per cubic centimeter}}{250,000}$$

MILLIGRAMS PER 100 CC. MEDIUM C	COUNT	MILLIGRAMS PER 100 CC MEDIUM C	COUNT
0	14		
0 5	16	50	45
1 0	16	100	74
5.0	18	125	64
10 0	21	150	101
20.0	31	175	94
30.0	26	200	115
40 0	29	250	102

TABLE 3

Summary of products formed by action of Aspergillus niger on glycerol and sucrose and upon those primary products

I	II	
Products formed from sucrose and glycerol	Products formed from compounds in column I	From
1. Acetic acid	Acetone	2
2. Citric acid	Citric acid	4
	Glycollic acid	1
3. Ethyl alcohol	Glyoxylic acid	1, 2
4. Gluconic acid	Malonic acid	2
	Oxalic acid	1, 2
5. Oxalic acid	Saccharic acid	4

In table 3 are listed the products reported as the result of the action of *Aspergillus niger* upon glycerol or sucrose together with those chemicals reported as due to the action of the mold on the primary products listed from glycerol or sucrose. The data are summarized from the review by Fulmer and Werkman (1930).

To none of the products listed may be attributed the stimulation of the yeast growth.

The stimulant above reported was an extracellular product. It seemed of interest to test the mold itself. A 200-gram sample of the dried mold was extracted for ten hours with 3 liters of water at 50 to 60°. The suspension was filtered and evaporated to a thick syrupy mass in vacuum and dried in a desiccator over $CaCl_2$.

TABLE 4

Yeast-growth stimulating properties of extracts of Aspergillus niger

Basal medium C for yeast. Count taken after forty-eight hours. Concentration of extract in milligrams dry material per 100 cc.

I		II		III		IV	
Concentration	Count*	Concentration	Count	Concentration	Count	Concentration	Count
0	41	0	50	0	37	0	38
0	35	0	48	0	38	0	38
0	37	0	54	0	38	0	37
5	57	2	50	2	67	2	49
10	74	4	49	10	112	10	77
20	119	10	52	20	106	20	74
30	116	20	56	40	148	40	120
50	162	40	56	60	114	60	106
60	141	60	55	80	99	80	115
80	159	80	55				

* Count = $\dfrac{\text{cells per cubic centimeter}}{250,000}$

I. Water-soluble fraction, pH of 1 per cent solution = 4.4.
II. Alcohol-insoluble fraction, pH of 1 per cent solution = 4.7.
III. Alcohol-soluble fraction, pH of 1 per cent solution = 3.3.
IV. Material removed from alcohol-soluble fraction by cooling to −10°C. pH of 1 per cent solution = 3.7.

The resulting material was quite black and dough-like in consistency. About 30 grams of residue were obtained. This is designated as fraction I. A 25-gram sample of this fraction was continuously extracted for four days with 95 per cent ethyl alcohol. The extract in contact with the mold was greenish in color while that in the reservoir was reddish brown. On cooling the alcohol extract to −10° a considerable quantity of needle-like

crystals separated out together with some tarry material. The alcohol-insoluble material is designated as fraction II. The alcohol-soluble material not removed by the cooling is fraction III and the material separated by cooling, fraction IV. The effects of these fractions upon the growth of yeast at 30° in medium C are shown in table 4. Fractions I, III, and IV are very rich in stimulant.

In table 5 are given data showing the production of yeast growth stimulant by *Trichoderma lignorum* and by *Aspergillus*

TABLE 5

Effect upon the growth of yeast of media which had supported the growth of Trichoderma lignorum and of Aspergillus clavatus

$$\text{Count} = \frac{\text{cells per cubic centimeter}}{250,000}$$

	TRICHODERMA LIGNORUM				ASPERGILLUS CLAVATUS			
	Sucrose medium		Glycerol medium		Sucrose medium		Glycerol medium	
	I	II	III	IV	V	VI	VII	VIII
Initial pH	6 9	6 9	6 9	6 9	6 9	6.9	6 9	6 9
	90	22	45	18	88	20	74	17
	83	23	42	15	83	13	77	19
	97	20	57	15	98	18	83	20
	88	18	46	14	110	17	72	23
	92	22			93	20	69	23
Final pH..........	3 10	5.85	5 0	5 5	3 0	5.5	3.4	5 75

I, III, V, VII had supported growth of mold; II, IV, VI, VIII, had not supported growth of mold.

clavatus on the sucrose and the glycerol media, using the same procedure as that employed in the studies on the production of the extracellular stimulant by *Aspergillus niger*.

SUMMARY

It has been shown that the growth of the molds *Aspergillus niger*, *Trichoderma lignorum* and *Aspergillus clavatus* either on glycerol or sucrose substrate produces in the medium a growth stimulant for *Saccharomyces cerevisiae*. Preliminary studies are reported on the bios content of *Aspergillus niger*.

REFERENCES

BUCHANAN, R. E., AND FULMER, E. I. 1930 Physiology and Biochemistry of Bacteria, vol. II, The Williams & Wilkins Company.

FULMER, E. I., AND HUESSELMANN, B. 1927 Iowa State Coll. J. Sci., 1, 411.

FULMER, E. I., AND NELSON, V. E. 1922 Jour. Biol. Chem., 51, 77.

FULMER, E. I., NELSON, V. E., AND SHERWOOD, F. F. 1921 Jour. Amer. Chem. Soc., 43, 186, 191.

FULMER, E. I., AND WERKMAN, C. H. 1930 Index to Chemical Action of Microorganisms, etc. C. C. Thomas.

NAYLOR, N. M., WEISBRODT-SMITH, L., AND COLLINS, H. J. 1930 Iowa State Coll., Jour. Sci., 4, 465.

TANNER, F. W. 1925 Chem. Rev., 1, 397–472.

MODIFICATION OF KOLLE FLASK

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Among the many difficulties encountered in studies requiring growth of bacteria in large quantities are those presented by the immense amount of glassware needed and the great space taken up by it in sterilizers and incubators. A simple modification of the Kolle flask which we have been using multiplies capacity by two, with a resultant economy of money and space.

The flasks are made with two indentations in the neck, one on each side (fig. 1). This makes it possible to use both sides and thus to make one flask do the work of two.

Agar is poured first onto one side, and—after this has hardened —onto the other. We have found the inoculation of such flasks from an aqueous suspension of bacteria to be a very simple matter and quickly accomplished. A convenient set-up for making the inoculations is shown in figure 2. By opening the pinch-cock (*a*) a few cubic centimeters of suspension are introduced into the flasks. This material is made to run over both surfaces of the agar by moving the flasks about in different directions.

These modified Kolle flasks appear to have no drawbacks. They are quite as useful as those made on the original model in case one wishes to use only one side. I am told by the manufacturer that, in quantity, they would cost no more to make. Unless, therefore, others find in them disadvantages which have not become apparent in our work it might be wise in the future, to have all Kolle flasks made with double instead of single indentations, so that either one or both sides could be used as desired. Such a change will only be feasible commercially if other bacteriologists indicate their wishes in the matter so that the manufac-
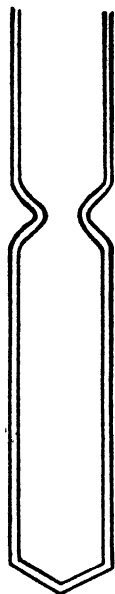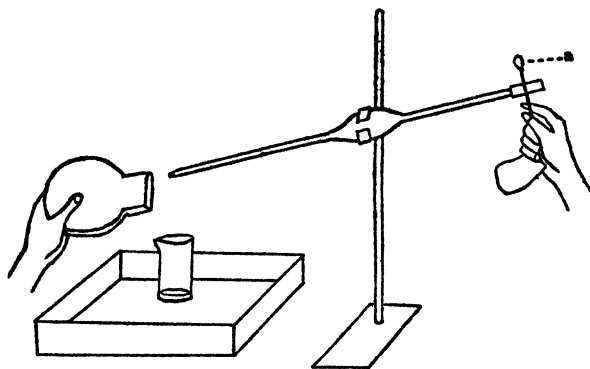
FIG. 1   FIG. 2

FIG. 1. CROSS-SECTION THROUGH LENGTH OF MODIFIED KOLLE PLATE

FIG. 2. A CONVENIENT SET-UP FOR INOCULATING MODIFIED KOLLE FLASKS FOR THE LARGE SCALE CULTIVATION OF BACTERIA. *A*, PINCH COCK

turers would feel justified in making the necessary small alterations in the mold. In order to interest others and thus to determine whether any wide need exists for these modified plates the suggestion is published here.

DETERMINATION OF CARBOHYDRATES IN BACTERI-OLOGICAL CULTURE MEDIA

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I. APPLICATION OF THE FERRICYANIDE REDUCTION METHOD TO QUANTITATIVE DETERMINATION OF GLUCOSE IN PEPTONE WATER

Introduction

In many quantitative studies of the carbohydrate metabolism of bacteria, it is more convenient to use small samples than large ones for analysis. On account of this fact, a reliable and accurate micro-method is more serviceable than a macro-method. At the outset of an investigation of glucose utilization by bacteria it was found unexpectedly that the micro-method in common use, based upon the reduction of copper, was subject to errors which have not been adequately considered by many who have employed this method. It was necessary, therefore, to reinvestigate micro-methods for the determination of glucose. The results of these studies, and of the application of a ferricyanide reduction method for the determination of glucose in peptone water and in bacterial cultures will be recorded in this communication.

Among the micro-methods, the volumetric copper reduction method of Shaffer and Hartmann (1920–21) has been used most frequently in bacteriological work for the determination of reducing sugars. Stiles, Peterson and Fred (1926) recommend the use of the micro-reagent of Shaffer and Hartmann, and present a table extending from 0.067 to 2.078 mgm., by which glucose may be determined from the difference in titration values obtained. The authors recommend the use of basic lead acetate to precipitate the protein, using disodium phosphate to remove the

excess lead.   Satisfactory results were obtained in some cases directly upon the medium without precipitation of the protein. As a test of the accuracy of the method the recovery of glucose added to media was determined.   The authors report "the agreement between the calculated and observed values varied between 0.01 and 0.04 per cent by the macro method.   Recovery by the micro method was fully as good or better than by the macro method."   By this is meant that when 1 per cent or 10 mgm. per cubic centimeter of glucose were added the glucose found was 0.99 per cent or 0.96 per cent (personal communication).   However, such a deviation at a concentration of 1 per cent or 10 mgm. per cubic centimeter means an error of 1 to 4 per cent, while the same deviation at a concentration of 0.5 per cent or 5 mgm. of glucose per cubic centimeter means an error of 2 to 8 per cent in the recovery of added glucose.

Magee and Smith (1930) using the Shaffer and Hartmann method for the determination of glucose in a meat infusion broth, report that an accuracy greater than ±10 per cent cannot be obtained.   The most accurate results were obtained directly upon the medium without precipitation of the protein.

Merrill (1930) reports the use of the Shaffer and Hartmann method as recommended by Stiles, Peterson and Fred.   The author says (p. 265).

Despite the fact that in making the media the glucose was accurately weighed and added in proportion of 1 gram made up to 100 cc. in volumetric flasks, values ranging from 0.95 to 1.06 per cent glucose were obtained by the Shaffer-Hartmann method.   It should also be noted that the same reagents were used throughout these determinations, a sufficient quantity being prepared in the beginning for all tests.   Thus, the maximum variation between the controls was approximately 10 per cent.   It seems justifiable to consider this the normal limit of variation of the carbohydrate determination.

Somogyi (1926) reporting a study of various copper reagents found that the reaction of the reagent is of extreme importance. He reports, "comparatively small changes in alkalinity produce considerable differences in reduction values" (p. 601).   The

changes in alkalinity are considered as affecting the breakdown of the glucose molecule into reactive fragments, rather than affecting the oxidizing power of the copper complex. He also calls attention to the danger of reoxidation of the cuprous oxide during the heating period. The reagent recommended is strongly buffered with a mixture of Na_2CO_3 and $NaHCO_3$.

The results I obtained in attempts to use the copper reduction micro-method of Stiles, Peterson and Fred (1926) were highly unsatisfactory. To summarize briefly the results obtained; no reagent could be prepared which would give 100 per cent recovery of glucose from aqueous solution when the table of Stiles, Peterson and Fred was used to compute results; the recovery of glucose added to media was variable at different times with the same reagent and varied with different reagents prepared at different times. Even upon solutions of pure glucose in distilled water, the determinations were subject to considerable variation. These variations could not be controlled by any means. The values obtained were closer to theoretical and showed less variation with smaller amounts of glucose (0.5 mgm. as compared to 1.0 mgm.). For a given concentration of glucose the values obtained showed less variation with a heating time of five minutes than with fifteen minutes. The variations, therefore, have been attributed to the effect of reoxidation of the cuprous oxide, during either the heating or cooling period.

In view of the complexity of the copper reagents, the known susceptibility of cuprous oxide to reoxidation and the irregular results obtained by various authors, it would seem advisable to replace the copper reduction method by one not possessing these disadvantages. The reduction of ferricyanide by sugars has often been reported in the literature, but until the present time, as far as is known to me, has not been used for the determination of reducing sugars in bacteriological media, with the exception of one article to which reference will be made later.

The Hagedorn-Jensen (1923a) ferricyanide method for the determination of blood sugar has been recommended by many workers. The principle of the method has been used by others who have extended the range, since the upper limit of the original

method is 0.385 mgm. Von Issekutz and von Both (1927) have modified the original method to cover a range from 0.725 to 14.99 mgm. of glucose. Bryant (1929) has modified the method for blood sugar determinations. Hanes (1929) has extended the range from 0.2 to 3.8 mgm. Sobotka and Reiner (1930) report satisfactory results with the method of Hanes. Gohr (1930) has successfully applied the reduction of ferricyanide to the determination of lactose in milk.

The publication of Cianci (1929) did not come to my attention until the data upon which the present paper is based had been practically completed. Cianci has applied the method of von Issekutz and von Both to the determination of glucose in an ammonium tartrate synthetic medium and in a peptone medium. The reduction of ferricyanide by bacterial bodies and by peptone was noted. The author believes that the determination of a correction factor for these reducing values gives more satisfactory results than any method attempting precipitation. Although Cianci is not clear as to the method of determining the correction factor and does not give the accuracy of the method, he is the first to publish the principle of the use of a correction factor for the purpose of obtaining greater accuracy in sugar determinations upon bacteriological media. The results upon synthetic media were extremely accurate being within 0.4 to 0.6 per cent of theoretical.

A brief review of the chemical principles upon which the method is based may be of value. Glucose, or another reducing substance, is heated with potassium ferricyanide in alkaline solution, the reduced ferrocyanide is precipitated by zinc sulphate in acid solution, while an amount of iodine equivalent to the remaining ferricyanide is liberated from potassium iodide and titrated with sodium thiosulphate.

(1) $K_3 [Fe(Cn)_6] + glucose \rightarrow K_4 [Fe(Cn)_6] + oxidation\ products$
(2) $H_4 [Fe(Cn)_6] + 2ZnSO_4 \rightarrow Zn_2 [Fe(Cn)_6] + 2H_2SO_4$
(3) $2H_3 [Fe(Cn)_6] + 2HI \rightarrow 2H_4 [Fe(Cn)_6] + I_2$
(4) $2Na_2S_2O_3 + I_2 \rightarrow Na_2S_4O_6 + 2NaI$

The reaction is carried to completion in all cases by the presence of the zinc sulphate which removes the ferrocyanide. A blank

without glucose is used to determine the amount of ferricyanide originally present. It is most convenient to express all values in terms of cubic centimeters of sodium thiosulphate. By subtracting the amount of thiosulphate solution required for the sample from that required for the blank, the number of cubic centimeters of thiosulphate equivalent to the ferricyanide reduced is obtained. Reference to a curve or table prepared from pure glucose solutions will give the amount of glucose or of reducing substance expressed as glucose, present in the sample.

II. THE FERRICYANIDE REDUCTION METHOD

To permit the use of a 50 cc. burette for titration it was necessary to employ less concentrated reagents than those recommended by Hanes (1929). The thiosulphate was reduced to 0.0050 normal solution and the concentration of the ferricyanide reagent adjusted so that large volumes of the thiosulphate would not be required for titration.

The modified reagents necessary for the determination are as follows:

1. $K_3 [Fe(Cn)_6]$, 4.2 grams; Na_2CO_3 (anhydrous), 10.6 grams. Dilute to 1000 cc. in a volumetric flask, using distilled water. If the solution is allowed to stand a week before using, the blank determinations will be constant, and will remain constant for several months. This solution should be stored in a brown glass bottle as it is affected by light.

2. KI, 25 grams; $ZnSO_4$, 50 grams; NaCl, 250 grams. Dilute in a volumetric flask to 1 liter with distilled water. Allow the solution to stand a few days then filter. This solution *must* be protected from light.

3. Five per cent acetic acid made by dilution of glacial acetic acid by volume.

4. Starch indicator: Dissolve 1 gram of soluble starch in 100 cc. of hot water. Add 20 grams of NaCl. This solution will keep indefinitely even though exposed to air.

5. Sodium thiosulphate: A tenth normal stock solution is prepared and kept on hand to be diluted for use whenever determinations are to be made. In making the stock solution the

addition of 0.1 to 0.2 gram of NaOH per liter of solution will aid in maintaining a constant normal factor over a long period of time. The stock solution should be standardized against a solution of KIO$_3$ or K$_2$Cr$_2$O$_7$. A convenient method of standardization is given by Hanes (1929). It is most convenient to adjust the stock solution to exactly 0.1000 normal, after which a dilution of 25 cc. to 500 or 50 cc. to 1000 will give an exactly 0.0050 normal solution. It has been found that more satisfactory results are obtained by titrating with a solution exactly 0.0050 normal than by titrating with one slightly stronger or weaker and converting to 0.0050 normal by use of the normal factor. It should be emphasized that the normal factor should be used for conversion if the thiosulphate varies by 0.0001 from 0.0050 since such a difference is sufficient to cause an error of about 2 per cent. After dilution the thiosulphate should be checked by titration against a suitable standard. It is well to keep two standard solutions for titration with the dilute thiosulphate and to check against each every few days.

The procedure for the determination is as follows: 1 cc. of the sample containing between 0.2 and 2 mgm. of glucose is measured into a 200 by 25 mm. Pyrex tube. Five cubic centimeters of the ferricyanide reagent are added followed by 4 cc. of distilled water, which is used to wash down the sides of the tube. The tubes are stoppered and heated for fifteen minutes in a vigorously boiling water bath, after which time they are removed and cooled by immersion in a beaker of cold water. Five cubic centimeters of the KI-ZnSO$_4$-NaCl reagent are added followed by 5 cc. of 5 per cent acetic acid and the liberated iodine is titrated immediately with 0.0050 normal sodium thiosulphate. The starch indicator (3 to 4 drops) is added when a faintly yellow color is still apparent, and the titration is continued to the disappearance of the blue color. The endpoint occurs as sharply as in any procedure which uses the starch-iodine titration although it may be seen a little better by diffuse daylight or artificial light than by direct sunlight. Due to the presence of the precipitate in the tubes it is necessary to shake them quite vigorously during the titration.

The stoppering of the tubes is not necessary to prevent re-

oxidation, but serves to prevent the entrance of water droplets during boiling. The time of cooling has no appreciable effect upon the values obtained, a fact previously óbserved by Bryant (1929).

The blank determination is carried out in the same manner upon 5 cc. of the ferricyanide reagent plus 5 cc. of water. For any given set of determinations the same pipette should be used for measuring out the ferricyanide reagent. It seems needless to say that all volumetric glassware, flasks for dilution, pipettes and burette, should be of suitable accuracy for quantitative work.

The amount of glucose present in the sample is obtained by subtracting the cubic centimeters of thiosulphate used for the titration of the sample from the cubic centimeters of the thiosulphate required for the blank and by reference to table 1 prepared from pure glucose solutions. This table of glucose equivalents is presented after considerable hesitation in view of the experience with tables prepared for copper reagents. The table originally prepared from more than 100 closely checking determinations has been repeatedly checked since then in the course of about a year's work, with many different preparations of the ferricyanide reagent and different samples of glucose. Although the blank determinations of two different reagents may differ by as much as 0.6 to 0.8 cc., the difference between titration values of blank and sample was always found to be the same.

The glucose used in the preparation of the table and in all subsequent work where a desired amount of glucose was added to media, was Pfanstiehl c.p. glucose which was kept over sulphuric acid in a vacuum desiccator for two weeks or longer before use. This fact must be recognized,—that the table is based upon anhydrous glucose,—since if glucose containing any moisture is used in making media, the results obtained by the determination of glucose upon a sample of the medium will indicate less than 100 per cent recovery of the added glucose. The use of anhydrous glucose gives an accurate standard to which other values may be compared by the reduction method.

As an illustration of the accuracy of the method upon pure glucose solutions the following example may be cited, in which

TABLE 1

Glucose-thiosulphate equivalents

Milligrams glucose equivalent to cubic centimeter thiosulphate difference in titration between sample and blank.

THIOSULPHATE	GLUCOSE	THIOSULPHATE	GLUCOSE	THIOSULPHATE	GLUCOSE
cc.	*mgm.*	*cc.*	*mgm.*	*cc.*	*mgm.*
1 0	0.181	4.6	0.832	8.2	1.484
1.1	0.199	4.7	0.850	8.3	1.502
1.2	0 217	4.8	0.868	8.4	1.520
1.3	0.235	4.9	0.886	8.5	1 538
1.4	0.253	5.0	0.904	8.6	1 556
1.5	0.271	5.1	0 922	8.7	1.575
1.6	0.289	5.2	0.940	8.8	1.593
1 7	0.307	5.3	0.959	8 9	1 611
1.8	0 325	5.4	0.977	9.0	1.629
1.9	0.343	5.5	0.996	9.1	1 647
2.0	0 361	5.6	1.014	9 2	1.665
2.1	0.379	5.7	1 032	9.3	1.683
2 2	0.397	5.8	1 050	9.4	1.701
2.3	0.415	5.9	1.068	9.5	1.719
2.4	0 433	6.0	1.086	9.6	1.737
2.5	0.451	6.1	1.104	9.7	1.756
2 6	0.469	6.2	1.122	9.8	1.774
2.7	0.487	6.3	1.140	9.9	1.792
2.8	0 505	6.4	1.158	10.0	1.810
2.9	0.524	6.5	1.176	10.1	1.828
3.0	0.543	6.6	1.194	10.2	1.846
3.1	0.561	6.7	1.212	10.3	1.864
3.2	0.579	6 8	1.230	10.4	1.882
3.3	0.597	6 9	1.249	10.5	1.900
3.4	0 616	7.0	1.267	10.6	1 918
3.5	0.634	7.1	1.285	10.7	1.936
3.6	0.652	7.2	1.303	10.8	1 954
3.7	0.670	7.3	1.321	10.9	1.972
3.8	0.688	7.4	1.339	11 0	1.991
3.9	0.706	7.5	1.357	11.1	2.009
4.0	0.724	7.6	1.375	11.2	2 027
4.1	0.742	7.7	1.393	11.3	2.045
4.2	0.760	7.8	1.411	11.4	2.063
4.3	0.778	7.9	1.429	11.5	2.081
4.4	0.796	8.0	1.448	11.6	2.099
4.5	0.814	8.1	1.466		

a reagent four months old was used. One cubic centimeter of a glucose solution containing 15.15 mgm. of glucose per cubic centimeter was diluted 1:10 in a volumetric flask. Glucose was determined upon duplicate 1 cc. samples of the dilute solution giving 1.502 and 1.511 mgm., average 1.506 mgm., difference from theoretical 0.009 mgm. or 0.6 per cent. A second dilution of the original sample gave 1.511 and 1.511 mgm.—difference from theoretical, 0.004 mgm. The variation between the averages of the two samples is less than 0.3 per cent. It is evident that the method shows a satisfactory accuracy upon aqueous solutions of glucose.

III. THE USE OF THE FERRICYANIDE METHOD FOR THE DETERMINATION OF GLUCOSE IN PEPTONE WATER

The peptone water used in these experiments was in all cases a 2 per cent aqueous solution of Bacto-peptone plus 0.5 per cent NaCl, sterilized by autoclaving at 15 pounds for fifteen minutes. Most of the samples were prepared in the usual manner in the media kitchen.

Since the method was designed for the determination of glucose upon 1 cc. quantities of a 1:10 dilution from an original sample of 1 cc., the dilutions in experimental work were made in the following manner: Stock solutions of glucose were prepared by accurately weighing the desired amount and diluting to volume with 0.5 per cent benzoic acid. It was necessary to add benzoic acid to prevent the growth of molds in the glucose solution kept in the icebox. The benzoic acid had no effect upon the reduction of ferricyanide. One cubic centimeter of the stock solution was pipetted into a 10 cc. volumetric flask, followed by 1 cc. of the peptone water under investigation. If a precipitant was used this was added, then after dilution to volume and mixing, 1 cc. samples were removed for the determination of reducing substance. This procedure provided a 1:10 dilution of the peptone water containing an amount of glucose dependent upon the concentration of the stock solution of glucose.

The accurate recovery of added amounts of glucose immediately presented difficulties. In all cases the determinations resulted in

more than 100 per cent recovery of glucose. The per cent recovery based upon the amount of glucose added increased as the actual amount of glucose present decreased. This pointed to the fact that the reduction by peptone was constant. A study of the reduction of the ferricyanide reagent by 1 cc. samples of peptone water showed that the amount of reduction was equivalent to 0.7 to 0.8 mgm. of glucose. If this reduction were neglected it would mean an error of 7 to 8 per cent on a 10 mgm. sample or 14 to 16 per cent on a 5 mgm. sample. Various precipitants among which may be mentioned tungstic acid, basic lead acetate and zinc hydroxide, were used in attempts to remove the reducing substance, but in no case could this be done.

On the other hand, it was found that the use of a precipitating agent was of value in the determination of glucose in cultures. Although none of the reducing substance is removed, the values upon individual determinations are slightly more constant after precipitation, and when the Somogyi (1930) zinc hydroxide precipitation is used filtrates from quite acid cultures are approximately neutral. Two other advantages of precipitation are the removal of bacterial substances which may exert an appreciable reducing action, and the fact that samples removed from a culture at intervals of time and immediately precipitated may be allowed to accumulate before a series of determinations are made. After precipitation of cultures no change in the reduction values takes place for at least twenty-four hours.

The method of precipitation used (except in the preliminary study of precipitating agents) and recommended is that of Somogyi (1930). The reagents and procedure are as follows:

(1) 10 per cent $ZnSO_4 \cdot 7H_2O$
(2) 0.5 per cent NaOH

The NaOH is so adjusted that 10 cc. of the zinc sulphate solution require from 10.8 to 11.2 of NaOH to produce a permanent pink color with phenolphthalein, the zinc hydroxide being titrated slowly with continuous shaking in the presence of 50 to 70 cc. of water.

For precipitation, 1 cc. of the sample (either sterile glucose-

peptone water or culture) is placed in a 10 cc. volumetric flask followed by 1 cc. of the zinc sulphate and 1 cc. of the sodium hydroxide. After dilution and mixing the sample may be filtered through a Whatman No. 1 filter paper into a small tube. No substances reducing ferricyanide were removed during the course of filtration.

The determination of the reducing substance of peptone could not be made upon 1.0 cc. samples of a 1:10 dilution because of the following phenomena. Although, theoretically, the reduction of 1 cc. samples of a 1:10 dilution should be one-tenth that of 1 cc. samples of the original peptone this is not the case. When the

TABLE 2

The effect of dilution upon the reducing action of peptone water

	TREATMENT	MILLIGRAMS AS GLUCOSE, EXTREMES 4 DETERMINATIONS	AVERAGE
Sample 1..........	Undiluted	0 817–0 869	0 847
	Diluted 1:10	1 020–1 036	1 030
Sample 2............	Undiluted	0 846–0 882	0.864
	Diluted 1:10	1 440–1 530	1 51
	Diluted 1:10 precipitated tungstic acid	1 44	1 44
	Diluted 1:10 precipitated $Zn(OH)_2$	1 260–1 620	1 44

values obtained upon 1:10 dilutions are calculated to the basis of reducing power per cubic centimeter of original peptone water, the result obtained is about 50 per cent greater than that obtained upon undiluted peptone water. This is shown in table 2, the values of the 1:10 dilution being calculated to the basis of undiluted peptone water for comparison. It will be noted that the variations between individual determinations are much greater than those obtained upon glucose solutions. The values obtained upon the undiluted peptone water correspond closely to those obtained by subtracting the added amount of glucose from the total reducing power as is done in the determination of the reducing constant which will be explained below. Although no attempt

is made to explain this phenomenon it should be noted that with the reagents used the values below 0.2 mgm. are more or less unsatisfactory from the standpoint of accuracy, although with glucose solutions the values obtained tend to be lower rather than higher than the theoretical values. The question of the nature of the reducing substance will be considered in the discussion.

In spite of these difficulties it has been found possible to determine glucose in peptone water with what appears to be a

TABLE 3

Reducing constant of peptone water

Determination of *rk* of 2 per cent peptone water and the recovery of added glucose by the use of the average *rk*.

GLUCOSE ADDED	TOTAL REDUCTION		*rk* TOTAL REDUCTION MINUS ADDED GLUCOSE	AVERAGE REDUCTION CORRECTED FOR AVERAGE *rk*	PER CENT RECOVERY OF ADDED GLUCOSE USING CORRECTED AVERAGE REDUCTION
	Duplicates	Average			
mgm.	*mgm./cc.*				
5.00	5 88– 5 61	5.75	0 75	5 05	101.0
	5 79– 5.70	5 75	0.75	5.05	101.0
	5.70– 5.52	5 61	0 61	4 91	98 2
	5.79– 5 79	5.79	0.79	5 09	101.8
15 15	15 93–15 66	15 79	0 64	15.09	99.6
	15.83–15 75	15.79	0.64	15.09	99 6
	15.83–15.83	15 83	0.68	15 13	99 8
Average			0.70		

satisfactory accuracy. This is made possible by the fact that the reducing power of a given sample of peptone water is constant. It is possible then to determine this constant reduction, for which the name reducing constant or *rk* is suggested. By the subtraction of the *rk* from the total reducing substance found in the medium after glucose is added or after growth has occurred, the amount of glucose is obtained.

Since this value must be determined upon the sterile medium before glucose is added the most convenient method for the preparation of a glucose-peptone water medium seems to be as follows:

the medium is prepared and sterilized in the usual manner, the rk is determined in the manner to be described, then using a 20 to 30 per cent solution of glucose *sterilized by filtration* the desired amount of glucose is added. A determination of the total reducing substance is made, the rk previously determined is subtracted, and the amount of glucose present is accurately known. The medium may be adjusted to any desired concentration by this

TABLE 4

The accuracy of recovery of added glucose using a previously determined rk

	PEPTONE SAMPLE	GLUCOSE ADDED	TOTAL REDUCTION, EXTREMES OF 4 DETERMINATIONS	AVERAGE	AVERAGE CORRECTED FOR rk	PER CENT RECOVERY CORRECTED
		mgm				
A	Not precipitated, rk = 0.6	10 03	10 59–10 83	10 65	10 05	100 1
			10 68–10 77	10 70	10.10	100 6
			10 50–10 83	10 71	10 11	100 7
			10 68–10 91	10 81	10.21	101.7
B	Not precipitated, rk = 0.7	8 09	8 69– 8 96	8 85	8.15	100 7
			8 69– 8 88	8 74	8 04	99.3
	Precipitated, Zn(OH)$_2$, rk = 0.7		8 69– 8 88	8.74	8 04	99 3
			8 51– 8 88	8 79	8.09	100.0
C	Not precipitated, rk = 0.8	8 09	8 78– 8 96	8 91	8 11	100.2
			8 87– 8 96	8 94	8 14	100 6
		5 02	5 79– 5.88	5 83	5 03	100 2
			5 88– 5 88	5 88	5 08	101 2
	Precipitated, Zn(OH)$_2$, rk = 0.8	10.23	10 86–11 13	10 97	10 17	99 4
			10 86–11.04	10 96	10.16	99 3
		10 00	11 86–10.95	10 86	10 06	100 6
			10 77–10 95	10.86	10 06	100 6

method. It is better to add a slight excess of glucose above the desired amount, and after determining how much is present dilute to the desired concentration with sterile peptone water having the same rk. The medium may then be distributed aseptically to suitable containers. Needless to say, the medium must be carefully guarded against changes in concentration due to evaporation. This procedure is not as laborious as it appears and seems to be the only way in which it is possible to make accurate glucose

determinations until some method is found for the precipitation of the reducing substances.

The determination of the rk of peptone water is made in the following manner: 1 cc. of a glucose solution of known concentration is placed in a 10 cc. volumetric flask, 1 cc. of the peptone water is added and precipitation carried out in the manner outlined above. The total reducing substance is determined upon 1 cc. quantities of the filtrate. Subtracting the milligrams

TABLE 5

The effect of the growth of various bacteria upon the non-precipitable reducing substance in peptone water. The recovery of glucose added after growth

CULTURE $rk = 0.7$	TUBE	GLUCOSE ADDED	TOTAL REDUCTION, DUPLICATES	TOTAL REDUCTION, AVERAGE	AVERAGE CORRECTED FOR rk	PER CENT RECOVERY USING CORRECTED AVERAGE
		mgm				
B. subtilis.........	1	10 00	10 68–10.59	10 63	9 93	99 3
	2	10 00	10 86–10 86	10 86	10.16	101 6
Staph. aureus	1	10 00	10 68–10 68	10.68	9 98	99.8
	2	10 00	10 68–10 86	10 77	10.07	100.7
B. aerogenes	1	10 00	10 68–10 95	10.81	10 11	101 1
	2	10 00	10 59–10.68	10 63	9.93	99 3
$rk = 0 8$			TOTAL REDUCTION, EXTREMES OF 4 DETERMINATIONS			
B. coli S.........	1	8 09	8 78– 8 96	8 91	8 11	100 2
	2	8 09	8 78– 8 87	8.85	8.05	99.5
	3	15 15	16 11–16 29	16 20	15 40	101.6
	4	15 15	15.85–16.02	15 95	15.15	100 0

of glucose added leaves the remainder as the reduction due to peptone expressed in terms of glucose. Several determinations should be made and averaged. The effect of the quantity of glucose added is negligible and may be disregarded when the total error of the method ± 2 per cent is accepted.

The values given in table 3 in milligrams per cubic centimeter refer to the calculated values of the original sample; the actual determinations were one-tenth of these values.

A large number of determinations have been made to study the recovery of added glucose from peptone water using the *rk* correction which had previously been determined on the sample. Table 4 gives a condensed summary of representative data obtained by this procedure, showing in all cases that the amount of glucose present in 2 per cent peptone water may be determined with an extreme error of ±2 per cent between the concentrations of 5 and 15 mgm. The recovery from 15 to 20 mgm., the upper limit of the method, is as good. Below 5 mgm. it would seem advisable to take a 2 cc. sample of the filtrate and after subtraction of twice the reducing constant calculate to the basis of 1 cc. of original sample. Further work is contemplated dealing with the lower region of the method.

A further step necessary to complete the method was to show that during growth the reducing substance of peptone water did not change. Table 5 shows that during the growth of the organisms used neither an increase nor a decrease of the *rk* took place. These data were obtained by determining the *rk* in the usual manner, then inoculating with the organisms concerned and determining the *rk* again after incubation. In all cases the recovery of glucose added after growth and corrected by the *rk* determined upon the sterile medium was within ±2 per cent, the accepted limit of the method. The data upon *B. coli* (table 5) were compiled from work done at different times. The data upon the other organisms (table 5) were obtained after four days of incubation. Determinations made, using several members of the paratyphoid group, gave the same results.

The method has been used successfully in the determination of the utilization of glucose by various species of bacteria in cultures in peptone water. The results of this study will be reported in a separate communication.

DISCUSSION

In view of the data presented, it is evident that the accurate determination of glucose cannot be made without the use of a correction factor for the reducing power of the medium. When this factor is taken into consideration and the method as outlined

above is used, it is possible to determine glucose within the limits of ±2 per cent upon bacterial cultures in a glucose peptone medium.

Attempts to apply the method to Douglas broth and media containing meat extract have thus far been unsuccessful because accurate correction factors could not be determined due to the fact that a part of the nonprecipitable reducing substance was utilized during the growth of the organisms. This factor does not enter into the work carried out upon peptone water since it has been demonstrated that the organisms used neither increased nor decreased the reducing substance in peptone water during twenty-four hours or longer of growth. The rk as determined upon a given sample before and after growth of these organisms, was constant within the limits of error of the method.

The nature of the substance or substances present in peptone water which reduce the ferricyanide reagent, is unknown. Attempts to isolate and concentrate this substance were unsuccessful. The fact that the Molisch reaction was always positive in filtrates which gave reduction, indicates the possibility of the carbohydrate-like nature of this substance. On the other hand, it is well known that the Molisch reaction is not specific for carbohydrates and the fact that the organisms used, in the aggregate having a great diversity of fermentative properties, did not appreciably attack the reducing substance indicates that it is not of a carbohydrate nature.

Furthermore, it is well known that the ferricyanide reagent is not specific for carbohydrates but is reduced by many other substances. Hagedorn and Jensen (1923b) reported that their reagent was reduced by uric acid and creatinine but not by either acetone or β-oxybutyric acid. Holden (1926) reported that uric acid, creatinine and cystine reduced the ferricyanide reagent while glutamic acid and glycine did not. No other substances were tried. Flatow (1928) reported the reduction of ferricyanide by uric acid, glutathione and thiasine. It is entirely possible that some of these substances may be present in the commercial products known as "peptone." However, the presence of none

could be conclusively demonstrated although it is highly probable that some free amino acids are present.

SUMMARY

A method is presented for the determination of glucose in peptone water, and bacterial cultures in this medium. This method is based upon the reduction of ferricyanide and involves the use of a correction factor for the reducing action of the peptone. It has been shown that the method possesses an accuracy of ±2 per cent.

REFERENCES

BENEDICT, S. R. 1926a The estimation of sugar in blood and normal urine. Jour. Biol. Chem., **68**, 759.

BENEDICT, S. R. 1926b The determination of blood sugar. II. Jour. Biol. Chem., **76**, 457.

BRYANT, H. L. 1929 A potassium ferricyanide method for the determination of reducing substances in blood. Jour. Lab. and Clin. Med., **14**, 1082.

CIANCI, V. 1929 Dosaggio del Glucosio Nelle Culture di Germi. Boll. della Societa Italiana di Biologia Sperimentale, **4**, 1136.

FLATOW, L. 1928 Über ferricyanometrische Mikromethoden in der Blutanalyse. Biochem. Zeit., **194**, 132.

GOHR, H. 1930 Titrimetrische Bestimmung der Lactose in der Milch. Zeits. f. Untersuch. der Lebensmittel, **59**, 90.

HAGEDORN, H. C., AND JENSEN, B. 1923a Zur Mikrobestimmung des Blutzuckers mittels Ferricyanid. Biochem. Zeit., **135**, 46.

HAGEDORN, H. C., AND JENSEN, B. N. 1923b Die Ferricyanidmethode zur Blutzuckerbestimmung. II. Biochem. Zeit., **137**, 92.

HANES, C. A. 1929 An application of the method of Hagedorn and Jensen to the determination of larger quantities of reducing sugars. Biochem. Jour., **23**, 99.

HOLDEN, H. F. 1926 The estimation of glucose in biological material. Biochem. Jour., **20**, 263.

VON ISSEKUTZ, B., AND VON BOTH, J. 1927 Einfache Methode zur Bestimmung der Glucose in Mengen von 1 bis 15 mg. Biochem. Zeit., **183**, 298.

MAGEE, M. C., AND SMITH, H. GREGG. 1930 A study of methods for the estimation of reducing sugars in bacteriological media. Jour. Bacteriol., **19**, 125.

MERRILL, M. H. 1930 Carbohydrate metabolism of organisms of the genus mycobacterium. Jour. Bacteriol., **20**, 235.

SHAFFER, P. A., AND HARTMANN, A. F. 1920–21 The iodometric determination of copper and its use in sugar analysis. II. Methods for the determination of reducing sugars in blood, urine, milk and other solutions. Jour. Biol. Chem., **45**, 365.

SOBOTKA, H., AND REINER, M. 1930 The Hagedorn-Jensen method applied to various sugars. Relation of reducing power to configuration. Biochem. Jour., **24**, 394.

SOMOGYI, M. 1926 Notes on sugar determination. Jour. Biol. Chem., **70**, 599.

SOMOGYI, M. 1930 A method for the preparation of blood filtrates for the determination of sugar. Jour. Biol. Chem., **86**, 655.

STILES, H. R., PETERSON, W. H., AND FRED, E. B. 1926 A rapid method for the determination of sugar in bacterial cultures. Jour. Bacteriol., **12**, 427.

THE SPECIFIC POTENCY OF CERTAIN CATIONS WITH REFERENCE TO THEIR EFFECT ON BACTERIAL VIABILITY[1]

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OBJECT OF STUDY

In previous contributions from this laboratory (Winslow and Hotchkiss, 1922; Hotchkiss, 1923; Winslow and Falk, 1923a; Shaughnessy and Winslow, 1927; Winslow and Dolloff, 1928; Fabian and Winslow, 1929) we have brought forward evidence to show that cations exert a highly characteristic effect upon bacterial viability.

The fact that a low concentration of a given salt stimulates biological action and a higher concentration inhibits it has been shown by numerous observers and in general all the studies have indicated much the same relative potencies of the various cations. Among the most important work along this line may be mentioned that of Lipman (1909) on the effect of NaCl, KCl, MgCl₂ and CaCl₂ upon ammonification by *B. subtilis*, of Brown and Hitchcock (1917) on nitrification in soils and of Brooks (1919, 1920, 1921) on carbon dioxide production by *B. subtilis*. Brown and Hitchcock (1917) present excellent curves for the influence of NaCl Na₂SO₄, MgSO₄, CaCO₃, NaHCO₃, Na₂CO₃ and CaCO₃ upon nitrification in soils. Here, however, calcium was least potent of the cations studied, in direct contrast with results in simpler media.

Brooks gives excellent curves for NaCl, KCl and CaCl₂ (1919) for MgCl₂ (1920) and for La (NO₃)₃ (1921) all showing stimulation of carbon dioxide production by low concentrations and inhibition

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[1] Based on a thesis presented by the junior author in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Yale University.

by higher concentrations.   Branham (1929) presents similar data for yeast.

In the first papers of our own series (Winslow and Hotchkiss, 1922; Hotchkiss, 1923) it was demonstrated that a wide variety of cations stimulate bacterial growth in low concentration and inhibit it in high concentration.   Winslow and Dolloff (1928) showed that the efficiency of each cation (both in the stimulating and in the toxic range) may be expressed by a characteristic constant and that mixtures of the chlorides of sodium, potassium and magnesium exhibit exactly the effects which would be predicated if their components acted in a purely additive fashion.   Fabian and Winslow (1929) found that the sodium ion exerted its characteristic effect in combination with a wide variety of anions including the hydroxyl ion, the result being determined by the combination of two factors,—concentration of sodium and pH.

It seems reasonable to conclude from these results and those of other workers that all cations exert upon bacterial viability a certain influence (aside from other more specific influences) which is qualitatively the same.   The quantitative effect of different cations varies very widely but each has a specific efficiency, both as regards stimulation and inhibition.   This characteristic, we propose to designate as "specific potency." The effect of mixtures of salts appears to be determined (aside from differences in pH) largely by the arithmetical sum of their specific potencies.

The present study was designed to test this postulate of specific potency by a careful study of salts and salt mixtures involving a larger group of cations than those reported upon by Winslow and Dolloff (1928).

<div align="center">TECHNIQUE</div>

The organism used in these studies was the same strain of *Escherichia coli (communis* type) used in all the previous work of this laboratory.   It was originally isolated from water in 1916 and is unusually well-adapted to such studies because it maintains itself in distilled water in almost undiminished numbers for a

period of 24 hours or more. The stock culture was maintained on nutrient agar with occasional passages through nutrient broth.

For the actual study of viability we used Dolloff's synthetic medium. This medium (Dolloff, 1926) consists of 5 grams of recrystallized ammonium tartrate, 5 grams of Pfanstiehl lactose and 0.02 gram of dibasic ammonium phosphate in a liter of water. Our stock solution was made up two and a half times this strength and sterilized at 15 pounds pressure for 20 minutes,—to be later added to the salt solutions to produce the standard concentration of the final medium.

The Dolloff medium was selected after a preliminary study in which it was compared with distilled water, used by Winslow and Falk (1923a) and with 1 per cent peptone water (Difco peptone) used by Hotchkiss (1923). It was expected that the activity of such salts as calcium might be very different in the different media since even so low a concentration as 0.005 M $CaCl_2$ formed a distinct precipitate in the Dolloff medium.

In distilled water there were on the average 11 million bacteria per cubic centimeter alive at the end of 48 hours, in Dolloff medium, 99 millions and in peptone-water, 161 millions. The quantitative effect of the salts tested (NaCl and $CaCl_2$) was to a slight degree affected by the medium, the nutrient materials present in the more complex media exerting a protective effect. Thus, maximum stimulation with NaCl was apparent at a concentration of 0.05 M in distilled water at 0.08 M in Dolloff medium and at 0.1 M in peptone-water. Marked toxicity appeared at 0.25 M in distilled water, and at 0.5 M in the other media. With $CaCl_2$, however, the effect was even less, optima so far as distilled water and Dolloff medium were concerned, being between 0.005 and 0.008 M in both cases and marked toxicity appearing at 0.01 M. In peptone-water, the toxic effect of $CaCl_2$ was markedly lowered, being insignificant even at 0.1 M. So far as the Dolloff medium was concerned, it seemed clear that such precipitation as occurred did not seriously affect the relative potency of the salts and this medium was therefore used in all succeeding work. In the inhibitive range many salts are rendered far less active in a peptone medium (Winslow and Dolloff, 1928) so that our results cannot be directly compared with those of Hotchkiss.

The salts used were all chlorides and were Baker Analyzed products. They were made up in convenient concentrations with sterile distilled water and stored in glass-stoppered bottles. All glassware, except that used for plating, was Pyrex and was allowed to stand at least twenty-four hours in cleaning solution, rinsed in hot water and in distilled water and sterilized at 180°C. for two hours.

In making our tests, the organism was grown for twenty-four hours on nutrient agar, washed from the slant with distilled water and then washed three times by centrifugalization. A suspension of the organism was then made up to contain approximately ten million organisms per cubic centimeter. Nine cubic centimeters of the test solution, containing a mixture of Dolloff medium and salt solution adjusted to give the desired final concentration, were inoculated with 1 cc. of this bacterial suspension, so that the initial concentration of organisms at the beginning of an experiment was about one million per cubic centimeter.

The suspensions thus prepared were incubated for 44 to 48 hours at 37°C., when plates were made in triplicate and colonies counted after 48 hours at 37°C.

The incubation period of 48 hours was selected after considerable preliminary experimentation with eight different salts. In these early studies the original suspension contained 20 to 50 million bacteria per cubic centimeter. In the Dolloff medium without added salts, the number rose to over 200 million after 24 to 48 hours and then fell to some 80 million after 144 hours. In favorable salt solutions the numbers rose to perhaps double their respective salt-free controls, while in unfavorable solutions the numbers fell off rapidly, in some cases reaching sterility after 48 hours. Stimulating effects were manifest in about the same degree at all the different time intervals (24, 48, 72, 96 and 144 hours); but slightly toxic salt concentrations tended to lose their inhibitive power after 48 hours, perhaps as a result of adaptation of the organisms to their menstruum. This phenomenon was marked in 0.05 M $CaCl_2$, 0.25 $MgCl_2$ 0.1 M LiCl and 0.0005 M $ZnCl_2$. For this reason, 48 hours was chosen as our standard test period since at this time the salt effects were most sharply contrasted.

Hydrogen ion determinations were made both before and after incubation by the electrometric method, using a Leeds and Northrup student's potentiometer with quinhydrone electrodes. Differences in reaction were not important under the conditions of this study. The Dolloff medium without added salts had a pH of 5.5 and remained at about that level. With the added salts the pH was a little higher, lying in the range 5.5 to 6.3 in 72 out of 80 experiments at the beginning and varying somewhat more widely at the end. Variations in bacterial numbers were not, however, correlated with differences in hydrogen ion concentration.

TABLE 1

Effect of various dilutions of NaCl upon viability of Es. coli in Dolloff medium

NaCl MOLALITY	BACTERIA IN MILLIONS PER CUBIC CENTIMETER									AVERAGE	PER CENT SURVIVAL AS COMPARED WITH SALT-FREE CONTROL	pH
1 0	0	0	0	0	0	0	0	0	0	0	0	5 1–5.6
0 5	59	26	30	4	75	65	3	28	28	42	37	5.4–5 7
0 25		129	154	146	79	130	104	161		129	115	5 5–6 1
0 10	142	108	164	172	147	182	147	110	202	153	137	5 5–6 4
0 08	119	164	195	191	182	149	179	133	240	172	154	5 5–6 8
0 05	97	87	226	224	81	115	151	92	191	140	125	5 5–6 9
0 01		178	173	231	74	134	82	125		142	127	5 5–6 6
0 005		202	166		66	132	79	114		126	113	5 6–6 8
0	67	87	185	157	119	136	95	49	114	112	114	5 5–5 5

EFFECTS OF THE NINE CATIONS STUDIED

The type of results obtained may be indicated by a single complete protocol presented in table 1. It will be noted that the number of duplicate determinations made at a particular dilution varied from 6 to 9 in this particular case. With many dilutions of other salts the number of duplicate determinations ran up to 10, 11 or 12. As in most bacteriological work, the variation between series is considerable but the general uniformity of the average results indicates that these random errors were reasonably well eliminated by the number of series averaged.

The average results, expressed for each concentration of each salt as a per cent of the number of bacteria present in the salt-free control, are presented in table 2 and in figure 1. All tests were made in Dolloff medium and the counts were made after 44 to 48 hours at 37°C.

TABLE 2

Survival of bacteria in salt solutions of various strengths as compared with salt-free control (per cent)

MOLALITY	NaCl	KCl	LiCl	BaCl₂	MgCl₂	CaCl₂	MnCl₂	ZnCl₂	CdCl₂
1 0	0	0							
0 5	37	91	0						
0.25	115	102	16	40					
0.1	137	115	51	54	56	36			
0 08	154		66		61				
0 05	125	155	111	123	87	62			
0 025		140	156	187	89				
0 01	127	127	121	199	119	159			
0 008					149	192			
0 005	113	104	93	170	140	176	15		
0.0025				162	128		25		
0 001				96	121	159	28	0	84
0 0008							46		
0.0005					114	142	87	57	87
0 00025							151	135	
0 0001						93	147	154	102
0.00008							137	208	104
0.00005							120	191	111
0 000025								142	121
0 00001								105	117
0.000005								86	91
0.000001									83

It will be noted from inspection of the table and the curves that all the cations studied show the same general phenomena. As the concentration of salt increases from a minimum, there is first an increasing stimulation of development (as measured by the count after 48 hours). As the salt is further increased, the numbers fall off again. This we have called the zone of decreasing stimulation. Finally, as the salt content becomes even higher we enter a zone of toxicity in which the number of bacteria is

below that of the control,—reaching a condition of sterility with
the highest salt concentrations. This is the zone of toxicity.
The point between the zone of diminishing stimulation and the
zone of toxicity where bacterial counts are approximately the
same as those of the salt-free control, we have called the cross-over
point.

For quantitative comparison of the individual cations we have
read off from the curves of figure 1 the concentrations correspond-
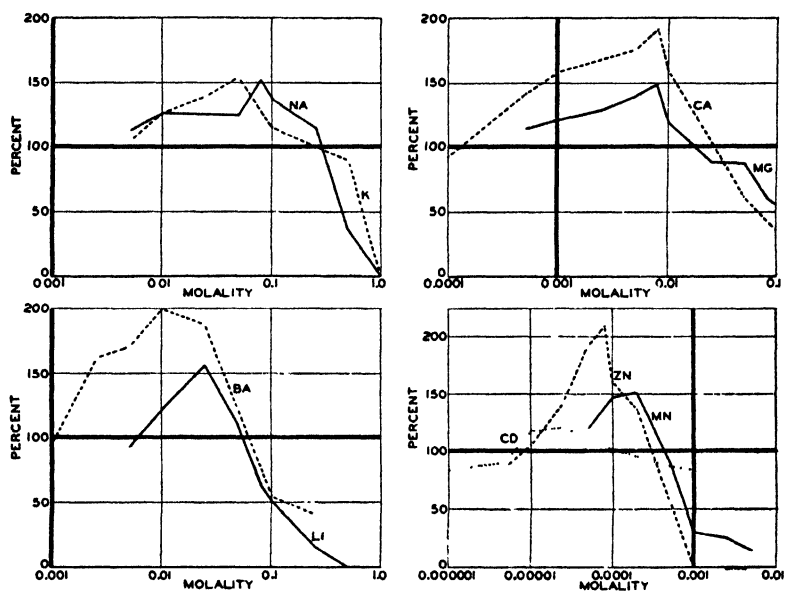
FIG. 1. RELATION BETWEEN SALT CONCENTRATION AND SURVIVAL OF BACTERIA IN
DOLLOFF SOLUTION AFTER 48 HOURS

Bacterial counts are expressed in percentages of the number present in a salt-
free control (abscissa corresponding to 0.001 molal solution indicated by heavy
line).

ing to counts equal to 125 per cent of the salt free control in the
zone of increasing stimulation, to the mid-point of the zone of
increasing stimulation, to the point of maximum stimulation, to
points corresponding to counts equal to 150 and 125 per cent
of the control in the zone of decreasing stimulation, to the cross-
over point and to points corresponding to 75 and 50 per cent
of the control in the zone of toxicity. The concentrations having

these comparable effects are presented in table 3. In computing the mid-point of the stimulating zone for NaCl, the abnormally low point at 0.05 M has been omitted.

It will be noted that the nine salts studied fall naturally into four groups, which are indicated in the four subdivisions of figure 1. The scales in these four subdivisions are the same but the actual points on the logarithmic abscissa are different, the abscissa corresponding to 0.001 M concentration being indicated by a heavy line in each instance. It will be noted that sodium and potassium

TABLE 3

Summary of molal concentrations of various salts producing certain effects upon bacterial viability

	ZONE OF INCREASING STIMULATION		MAXIMUM	ZONE OF DECREASING STIMULATION		CROSS-OVER POINT	ZONE OF TOXICITY	
	Percentage of salt-free control							
	125	Midpoint 111-154	121-208	150	125	100	75	50
Na	0 009	0 01	0 08	0 09	0 17	0 28	0 36	0.44
K....	0 009	0 01	0 05	0 05	0 08	0 29	0 56	0 68
Li	0 01	0 01	0 02	0.03	0 04	0 06	0 07	0.10
Ba	0 001	0 002	0 01	0 04	0 05	0.06	0 08	0.13
Mg...	0 002	0 002	0 008	0 008	0 01	0 02	0 07	
Ca	0 0003	0 0006	0.008	0 01	0 02	0 03	0.04	0 07
Mn........	0 00006	0 00006	0 0002	0 0003	0 0003	0 0004	0 0006	0 0008
Zn	0 00002	0 00004	0 00008	0 0001	0 0003	0 0003	0 0004	0 0005
Cd....		0 000008	0 00002			0.0001		

are essentially identical in their effect. Barium and lithium are effective in lower dilution (both in stimulation and toxicity), their curves lying to the left of those for sodium and potassium. Calcium and magnesium form a still more potent pair and manganese, zinc and cadmium are some hundreds of times more powerful than sodium and potassium, their curves lying almost wholly below the 0.001 M concentration, while the curves for the other cations studied lie almost wholly above this point.

For the most part, the results obtained check fairly well with those obtained in earlier studies.

The point of maximum stimulation for NaCl was found at 0.08 M while the stimulating range lay between 0.005 M and 0.25 M. These results correspond with those of Winslow and Falk (1923a) who found 0.01 NaCl favorable and 0.7 M NaCl toxic and with those of Winslow and Dolloff (1928) who found a range of stimulation between 0.0001 M and 0.3 M with a maximum at 0.05 M. Fabian and Winslow (1929) reported stimulation extending up to 0.3 M with a maximum at 0.1 M. Hotchkiss (1923) found a higher concentration associated with maximum growth (0.25 M) but, as pointed out above, the peptone medium and the 3-day incubation period which she used require a higher salt concentration to yield a given result.

With KCl, the stimulating range is the same as that for NaCl (0.005 to 0.25 M), the maximum falling at 0.05 M. This maximum point is lower than that recorded by either Winslow and Dolloff (0.2 M) or Hotchkiss (0.25 M).

The stimulating zone for LiCl begins at about the same point as that of Na and K (0.006) but is much narrower, having its maximum at 0.025 M and ending at about 0.05 M, beyond which the salt is toxic. These values as usual are slightly lower than those of Hotchkiss.

The stimulating zone for $BaCl_2$ begins at a very low concentration (0.001 M), has a maximum at 0.01 M and ends about where that for Li does, at 0.06 M. This corresponds closely with the Hotchkiss figures.

$MgCl_2$ and $CaCl_2$ are closely alike. Both have stimulating zones beginning near 0.0001 M with maxima at 0.008 M and extending to about 0.02 M, beyond which point both become toxic. For Mg, these figures correspond with those of Winslow and Dolloff who found maximum stimulation at 0.003 M. As usual, Hotchkiss' results in peptone solution show a limitation of salt potency, the range 0.0025 to 0.1 M being stimulating in her study. For calcium, the constants reported by various observers differ more widely than for any other salt. Thus, Hotchkiss reported maximum stimulation at 0.15 M and stimulation up to 0.25 M while our maximum is at 0.008 M and our cross-over point at 0.03 M. It is true that the peptone medium and the long incubation may

account for this but Winslow and Falk (1923a) using water found a cross-over point at 0.14 M and Winslow and Dolloff (1928) one at 0.1 M.

MnCl$_2$ is stimulating between perhaps 0.00003 and 0.0004, with a maximum at 0.0002. This corresponds with the Hotchkiss results as does the curve for ZnCl$_2$, showing stimulation between 0.00001 and 0.0003, with a maximum at 0.00008, CdCl$_2$, with a stmulating range between 0.00001 M and 0.0001 M, was less toxic than reported by Hotchkiss.

TABLE 4

Specific potency of various cations

	ZONE OF INCREASING STIMULATION		MAXIMUM	ZONE OF DECREASING STIMULATION		CROSS-OVER POINT	ZONE OF TOXICITY		AVERAGE
	Percentage of salt-free control								
	125	Midpoint		150	125	100	75	50	
Na....	1	1	1	1	1	1	1	1	1
K	1	1	1 6	1 8	2 1	1 0	0 6	0 6	1 2
Li	0 9	1	4 0	3 0	4 3	4 7	5.1	4 4	3 4
Ba........ ..	9	5 0	8 0	2 3	3 4	4 7	4 5	3 4	5 0
Mg........ ...	4 5	5 0	10	11	17	14	5.1		9 4
Ca.............	30	17	10	9 0	8 5	9 3	9.0	6 3	12 0
Mn...	150	170	400	300	600	700	600	600	400
Zn....	450	250	1,000	900	600	900	900	900	700
Cd....		1,200	4,000			2,800			3,000

On the whole these results seem reasonably consistent with earlier findings and the relative order of the salts from the standpoint of both stimulating and inhibiting effects, is the same in all studies.

In order to bring out more clearly the relative effect of the individual cations indicated in table 4 we have computed their specific potencies for each of the points included in table 3 as reciprocals of the ratios of the concentration of a given salt to the amount of NaCl necessary to produce the same effect.

The average specific potencies are, then, as follows, taking the potency of Na as 1; K, 1.2; Li, 3.4; Ba, 5.0; Mg, 9.4; Ca, 12; Mn, 400; Zn, 700; Cd, 3000.

It seems clear from the table that the concept of a specific potency, characteristic of each cation, is a valid one. The explanation of this phenomenon is still obscure; but it seems highly probable that the action of the cations may be explained on Bancroft's theory of disinfection (Bancroft and Richter, 1931) as related to coagulation of colloids. The effects of cations upon the coagulation of such colloids as sulphur and mastic show specific potencies for the various cations varying in somewhat the same orders of magnitude (Bancroft, 1921) although the relative potency of the various cations is widely different from that observed by us. Bancroft, however, states that in such coagulations "the fundamental rule is that the adsorption is specific both as regards the adsorbing substance and the ion adsorbed."

In the Winslow and Dolloff study comparison was made not on the basis of molality but of ionic activity. Similar computations were made in the present study, using the tables of Lewis and Randall (1923). The differences in specific potency as computed on the basis of molality and of ionic activity did not, however, differ materially. The figures for calcium and magnesium and barium were slightly increased, those for zinc and cadmium slightly decreased. Since the differences were insignificant and the application of the Lewis and Randall constants seems of doubtful validity in the relatively complex medium used, we have considered comparison on the basis of molality the soundest basis available.

EFFECTS OF MIXTURES OF THE CATIONS STUDIED

As a check on the soundness of the theory of specific potencies we planned a second series of experiments in which mixtures of various salts were prepared and the effect upon bacterial viability determined in order to see whether the actual results would conform to those predicted from the theory. Five concentrations of each salt were chosen, which lay on the descending side of the curves of figure 1 and which would by themselves give counts corresponding to 150 and 125 per cent of the salt-free control in the zone of diminishing stimulation, to the cross-over point, and

to 75 and 50 per cent of the control in the zone of toxicity. For NaCl and KCl five mixtures were prepared as follows; one containing the 150 per cent concentration of NaCl (0.09 M) and the 50 per cent concentration of KCl (0.68 M); one containing the 125 per cent concentration of NaCl (0.17 M) and the 75 per cent concentration of KCl, (0.56 M); one containing the cross-over

TABLE 5

Survival of bacteria in salt mixtures expressed as per cent of number in salt-free control

Bacterial count corresponding to concentration of	NaCl	150	125	100	75	50
	Second salt	50	75	100	125	150
SALTS PRESENT						
NaCl, KCl...		84	87	100	109	124
NaCl, LiCl		87	91	99	83	86
NaCl, BaCl₂...		108	97	97	98	87
NaCl, MgCl₂............		117	103	113	111	105
NaCl, CaCl₂		117	**133**	109	108	91
NaCl, MnCl₂		**65**	89	98	92	90
NaCl, ZnCl₂		86	87	88	94	94
Bacterial count corresponding to concentration of	CaCl₂	150	125	100	75	50
	Second salt .	50	75	100	125	150
CaCl₂, KCl.............		108	109	105	125	98
CaCl₂, LiCl		**46**	**59**	**67**	77	**68**
CaCl₂, BaCl₂		**167**	**155**	**155**	**135**	118
CaCl₂, MgCl₂		84	97	89	88	96
CaCl₂, MnCl₂........ ...		103	108	101	99	84
CaCl₂, ZnCl₂		**43**	**65**	**37**	**49**	**39**

Figures which deviate by more than 25 per cent from the expected value are boldface.

concentration of each salt (Na, 0.28 M, K 0.29 M); one containing the 75 per cent concentration of NaCl (0.36 M), and the 125 concentration of KCl (0.08 M) and one containing the 50 per cent concentration of NaCl (0.44 M) and the 150 per cent concentration of KCl (0.05 M). In each case the two solutions were mixed in equal proportions so that if the theory of specific potencies held, and no other

phenomena intervened, the final counts should in all cases approximate that of the salt-free control. According to the theory a half and half mixture of two equipotent salts should produce the same effect as either undiluted salt alone and a similar mixture of two salts having equal but opposite effects should have the same effect as a solution of either salt of a strength half way between the two extremes. Mixtures were made in this way of NaCl with each of
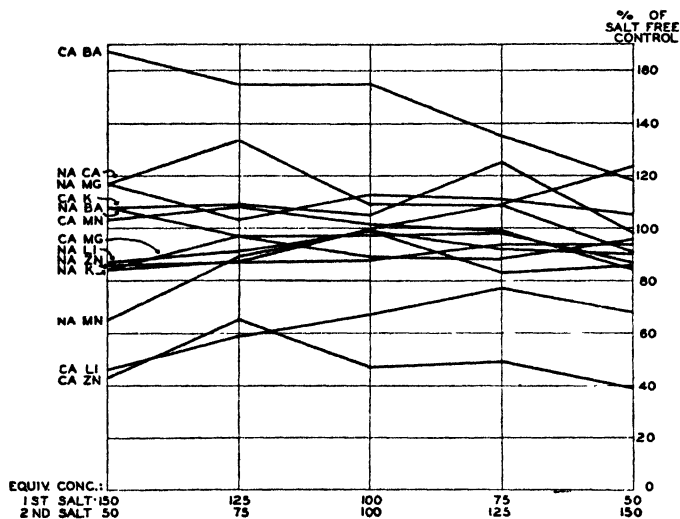
FIG. 2. SURVIVAL OF BACTERIA IN VARIOUS SALT MIXTURES EXPRESSED AS PER CENT OF NUMBER IN SALT-FREE CONTROL

In each experiment a solution of sodium or calcium chloride was mixed with a solution of the chloride of some other cation. The upper line of figures at the bottom of the chart represents the relative count which would be obtained from the original solution of the first salt (sodium or calcium chloride); the lower figure represents the count which would be obtained in presence of the original concentration of the second salt used.

the other salts and of CaCl₂ with each of the other salts (except CdCl₂) and 5 to 7 duplicate determinations were made for each mixture. The average results are summarized in table 5 and in figure 2.

Before discussing the results presented in table 5 it should first be made clear just what effect would be expected from the single salts used in these mixtures if acting alone. The "Bacterial counts corresponding to concentrations of salt" which head table

5 refer to the counts obtained from the solutions before mixture but mixture of course diluted each one-half. When we mixed cross-over concentrations of NaCl and CaCl$_2$, for example, each of the solutions by itself would have produced a count equal to the control. The actual solutions mixed were 0.28 M NaCl and 0.02 M CaCl$_2$. In mixing, however, each of these solutions was diluted one-half. The actual amount of NaCl used (0.14 M) would by itself have produced a count of 130 per cent of the control and the actual amount of CaCl$_2$ (0.01 M) a count 135 per cent of the control. The individual diluted salt solutions reviewed in table 5 would in all but six instances by themselves have produced stimulation. Thus, a mixture of two stimulating salts in these experiments produces no stimulation. This is the sort of phenomenon often described as antagonism, but it is clearly only an additive effect of two different cations, each exerting its independent specific potency effect.

According to our theory, on the other hand, the effect of a salt mixture should be predicted by multiplying the concentration of each cation by its specific potency and then adding the two results. Thus, in the mixtures of NaCl and CaCl$_2$ for example the total specific potencies in terms of Na would be as follows; corresponding to the five columns in table 5.

Most stimulating Na, most inhibitive Ca:
   0.045 M Na + 12 × 0.035 M Ca = 0.46 M Na
Stimulating Na, inhibitive Ca:
   0.085 M Na + 12 × 0.02 M = 0.32 M Na
Neutral Na, neutral Ca:
   0.14 M Na + 12 × 0.015 M Ca = 0.32 M Na
Inhibitive Na, stimulating Ca:
   0.18 M Na + 12 × 0.01 Ca = 0.30 M Na
Most inhibitive Na, most stimulating Ca:
   0.22 M Na + 12 × 0.005 Ca = 0.28 M Na

Thus, the combined effect of the two salts in the mixture should be equal to a NaCl of about 0.3 M strength which would correspond on the Na graph of figure 1 to a count of about 90 per cent of the salt-free control.

Similarly, we may compute the same salt combinations in terms of Ca by dividing each actual concentration of Na by 12.

This gives for the five points total potencies corresponding to 0.023, 0.025, 0.027, 0.027, and 0.039 M $CaCl_2$ respectively an average of 0.028 Ca, a concentration of the calcium curve in figure 1 corresponding to a count just equal to the salt-free control.

Such combined potencies have been computed for all of the sixty-odd salt mixtures used and most of them give results lying close to the cross-over concentrations of Na and Ca. Returning to table 5 we note that the results for mixtures of NaCl with other salts are about what would be expected from the specific potency theory. There are only two figures in the upper half of the table which fall above 125 or under 75 per cent. These exceptions, which are boldfaced in the table, are a high value for one mixture of NaCl and $CaCl_2$ and a low value for one mixture of NaCl and $MnCl_2$.

The mixtures of $CaCl_2$ with KCl, $MgCl_2$ and $MnCl_2$ also run close to expectation. The mixtures of $CaCl_2$ with LiCl and with $ZnCl_2$ on the other hand show very low values throughout and the mixtures of $CaCl_2$ with $BaCl_2$ show very high values throughout. The latter may perhaps have been influenced by precipitation but the low values with Li and Zn are puzzling and suggest some action differing from the usual specific potency effect. We have at present no explanation to offer for this phenomenon.

SPECIFIC POTENCY AND SALT ANTAGONISM

The peculiar effect of mixtures of $CaCl_2$ with LiCl, $BaCl_2$ and $ZnCl_2$ furnishes a salutary warning against any generalization which tends to over-simplify the phenomena of salt action. Yet the general validity of the specific potency principle seems established and it is tempting to speculate as to the extent to which this principle may explain the effect described as "salt antagonism."

The concept of salt antagonism implies a specific neutralization by one salt of the effect of another. It seems beyond question to occur when the cells and tissues of animals, such as starfish eggs and mammalian muscle tissue, are exposed to salt mixtures.

In interpreting the effect of salt mixtures upon bacteria the phenomena of specific potency must however be kept in mind,

and we must also be quite clear as to the difference between mixing two salt solutions of known strength (which involves dilution of each) and the addition of solid salt to a solution of another salt (which does not involve dilution). It is obvious that if we take two different concentrations of the same salt and mix them the effect will be the same as that of an intermediate concentration. What that result will be, however, will depend on the particular part of the potency curve at which the concentrations used may lie. Thus from figure 1, it appears that if we mix two concentrations of NaCl, both of less than 0.05 M strength, we shall get a stimulating effect intermediate between that of the two concentrations used (since both lie in the zone of increasing stimulation). If, however, we mix a concentration lying in the zone of increasing stimulation (say 0.01 M) with a concentration in the zone of decreasing stimulation (say 0.1 M) we shall obtain a greater stimulation than that given by either primary concentration alone since the mixture will correspond to the point of maximum stimulation. If we mix a concentration lying in the zone of diminishing stimulation (say 0.1 M) with a concentration in the zone of toxicity (say 0.44 M) we shall obtain a neutralization of effects. The last two are just the sort of phenomenon often described as antagonism when two salts are used. Yet with one salt alone it is clearly not antagonism but addition which is taking place. The results reported in preceding pages like those of Winslow and Dolloff (1928) show that when different salts are used the phenomena often follow the same law and, when they do so, the assumption of antagonism is superfluous.

In other studies of so-called antagonism, instead of mixing two salt solutions (and thus diluting each) a second salt is added to a solution of the first salt, keeping the concentration of the first unchanged. Here the problem is simpler but the result will still be largely determined by the part of the specific potency curve in which the addition takes place.

Thus, if we start with a salt concentration lying in the zone of increasing stimulation the addition of a small amount of another cation will push the total cation concentration up to the point of maximum stimulation. A larger addition will carry the total

concentration over to the zone of decreasing stimulation or the zone of toxicity. If we start with a salt concentration giving maximum stimulation the addition of any other cation will carry the total concentration into the zone of decreasing stimulation or toxicity; so that starting at this point any salt will appear antagonistic to any other salt, even if the concentration of the second salt added were itself stimulating in effect.

The only thing that cannot occur according to the principle of specific potency is the neutralization of toxic effect by the actual addition of any cation to a solution already toxic (without dilution). This is the critical test, since according to the theory of antagonism each cation exerts its characteristic effect uninfluenced by the other while according to the uncomplicated effects of specific potency, the addition of any amount of a second cation should increase the toxic effect of the first.

One of the clearest cases of such true antagonism was presented by Winslow and Falk (1918) with regard to Na and Ca. Solutions of 0.6 M NaCl and 0.1 M $CaCl_2$ were highly toxic but a solution containing both these salts in the same concentration was nontoxic. In a later paper the same authors showed that this phenomenon only occurred in alkaline solutions and was related to the influence of calcium on the power of the cell to buffer the adjacent medium, which influence was eliminated by NaCl (Winslow and Falk, 1923b).

Eisler (1909) also reports what seems to be true antagonism. He found 0.1 M solutions of LiCl to be toxic to *B. subtilis* and could abolish this toxicity by further addition of 0.05 M $CaCl_2$, 0.005 M $BaCl_2$, 0.02 M $Ca(NO_3)_2$ and 0.005 $MgSO_4$ but not by addition of K_2SO_4. Toxicity of 0.0015 M solutions of $MnSO_4$ was also abolished by 0.001 M $Ca(NO_3)_2$ and 0.01 KCl. This certainly looks like true antagonism such as has been described among the higher organisms.

The work of Lipman (1909) on ammonification by *B. subtilis* also shows true antagonism, since inhibiting concentrations of KCl and NaCl were made favorable by the addition of small amounts of $CaCl_2$ and $MgCl_2$ respectively while mixtures of 20 parts of NaCl to one of KCl and of 2 parts of KCl to one of NaCl

were more favorable than either salt alone. He found no antagonism between calcium and magnesium or between sodium and calcium (Lipman, 1910).

Elaborate studies on this problem of salt antagonism in the botanical field have been carried out by Osterhout and his pupils, using the electrical resistance of Laminaria as a criterion of physiological effects. His earlier work was with NaCl and $CaCl_2$ (Osterhout, 1912, 1914) and led to the general conclusion that certain salts like NaCl increase permeability while others, like $CaCl_2$ decrease it (with an ultimate subsequent increase); and that antagonism exists when we mix substances of these two types (Osterhout, 1915). Later, a chemical formula was devised to predict the observed results (Osterhout, 1917).

In later studies with bile and alkaloids, Osterhout (1919) added cevadine sulphate for example to a solution of NaCl which by itself greatly increased permeability. The addition of increasing amounts of the alkaloid caused first a rise in permeability and then a fall. This again may be a purely additive effect if it be assumed that the principle of specific potency applies to effects on higher plant tissues, since an additive effect would first replace decreased by increased permeability and then, with further addition, would cause the secondary increase in permeability associated with very high salt concentrations (Shaughnessy and Winslow, 1929). The crux of the matter is whether an increase in NaCl alone would produce the same results as the addition of the second substance.

A particularly interesting series of studies were made by Brooks (1919, 1920, 1921) in Osterhout's laboratory on the effect of salts upon carbon dioxide production by B. subtilis and by Gustafson (1919) on the carbon dioxide production of Aspergillus, Brooks (1919) shows for NaCl, KCl and $CaCl_2$ curves of specific potency exactly like those later described by us, with stimulation by $CaCl_2$ at 0.05 M, by NaCl at 0.15 M and by KCl at 0.2 M and decrease in carbon dioxide production at higher concentrations. She finds marked antagonism between NaCl and $CaCl_2$ and between KCl and $CaCl_2$ with slight bimodal antagonism between NaCl and KCl. Similarly, antagonism has been shown between NaCl

and $MgCl_2$ (Brooks, 1920) and between NaCl and La $(NO_3)_3$ (Brooks, 1921). All these experiments were made by using solutions of equi-molality but varying the proportion of the two constituents; and at the point where Brooks finds stimulation (with NaCl and $CaCl_2$ for example) the concentration of NaCl in the mixture would by itself be markedly inhibitive so that its neutralization by $CaCl_2$ is true antagonism.

The results of Shearer (1917, 1920) seem at first to show true antagonism. He found that dilute solutions of monovalent salts (Na, K, Li) produced a rapid increase in electrical conductivity in a suspension of the gonococcus with death of the bacteria, which process was checked by the addition of bivalent ions. We are here, however, dealing with a phenomenon quite different from that of the viability of *Es. coli* in Dolloff medium. The colon bacillus grows in Dolloff medium and dilute salts stimulate its growth by increasing permeability (Shaughnessy and Winslow, 1927). The gonococcus in salt solution does not grow but merely tends to autolyze and increased permeability increases this tendency. Therefore dilute salts which favor *Es. coli* should be unfavorable to the gonococcus. The use of stronger cations (Ca, Ba, Cd), the addition of these bivalent ions to NaCl, or the use of a stronger NaCl solution, all should neutralize the effect of dilute univalent ions and check the increased permeability,— favoring survival. All of these results were obtained by Shearer and are clearly explicable on the theory of the additive effects of specific potency. The bivalent ions in the concentration used by Shearer caused a temporary rise in resistance followed by a slow but irreversible fall which corresponds to the effects on permeability recorded by Shaughnessy and Winslow. There is therefore no evidence of antagonism in the sense of a characteristic opposite effect of univalent and bivalent ions but merely the specific potency of each cation in its effect on permeability exerted by the cations in just the same way when alone or in combination.

It appears, then, that some of the phenomena described as "salt antagonism" can be explained on the simpler ground of additive effects of specific potencies. Others, like the observations of Winslow and Falk, Lipman, Osterhout and Brooks, can not.

SUMMARY OF CONCLUSIONS

Whatever more complex and specific effects may be superimposed thereupon, there seems to be a very fundamental and general influence exerted by all cations upon the bacterial cell. This influence normally takes the form of stimulation of viability (associated with increased permeability) when the cation is present in low concentration and of inhibition (associated with decreased permeability) in higher concentration. The cations vary widely in quantitative efficiency, each one having a "specific potency" of its own which is roughly constant in relation to the specific potency of other cations both in the stimulating and in the inhibiting zone. If the specific potency of Na be taken as 1, that of the other cations here studied may be approximately stated as follows: K, 1; Li, 3; Ba, 5; Mg, 9; Ca, 12; Mn, 400; Zn, 700; Cd, 3000. Some, but not all, of the phenomena of salt antagonism can be explained as due to a simple additive effect of the specific potencies of the cations concerned.

The phenomena of specific potency seem to fit rather satisfactorily into Bancroft's theory of disinfection (Bancroft and Richter, 1931) according to which disinfection (like narcosis in the higher forms of life) is due to coagulation of cell colloids, the decreasing stability of these colloids in the initial stages of coagulation being associated with stimulation. Only when due allowance has been made for specific potency effects can antagonism be postulated.

REFERENCES

BANCROFT, W. D. 1921 Applied Colloid Chemistry. New York.
BANCROFT, W. D., AND RICHTER, G. H. 1931 Jour. Phys. Chem., **35**, 511.
BRANHAM, S. E. 1929 Jour. Bact., **18**, 247.
BROOKS, M. M. 1919 Jour. Gen. Physiol., **2**, 5.
BROOKS, M. M. 1920 Jour. Gen. Physiol., **3**, 331.
BROOKS, M. M. 1921 Jour. Gen. Physiol., **3**, 337.
BROWN, P. E., AND HITCHCOCK, E. B. 1917 Soil Science, **4**, 207.
DOLLOFF, A. F. 1926 Science, **64**, 254.
EISLER, M. 1909 Centr. f. Bakt. Abt., **51**, 546.
FABIAN, F. W., AND WINSLOW, C.-E. A. 1929 Jour. Bact., **18**, 265.
FALK, I. S. 1923 Abst. Bact., **7**, 33.
GUSTAFSON, F. G. 1929 Jour. Gen. Physiol., **2**, 17.
HOTCHKISS, M. 1923 Jour. Bact., **8**, 141.

LEWIS, G. U., AND RANDALL, M. 1923 Thermodynamics and the free energy of chemical substances. New York.

LIPMAN, C. B. 1909 Bot. Gaz., **48**, 105.

LIPMAN, C. B. 1910 Bot. Gaz., **49**, 41.

OSTERHOUT, W. J. V. 1912 Science, N.S., **35**, 112.

OSTERHOUT, W. J. V. 1914 Jahrb. f. wiss. Bot., **54**, 645.

OSTERHOUT, W. J. V. 1915 Science, N.S., **41**, 255.

OSTERHOUT, W. J. V. 1917 Scence, N.S., **45**, 97.

OSTERHOUT, W. J. V. 1919 Jour. Gen. Physiol., **1**, 515.

SHAUGHNESSY, H. J., AND WINSLOW, C.-E. A. 1927 Jour. Bact., **14**, 69.

SHEARER, C. 1917 Proc. Roy. Soc. London, Series B, **89**, 440.

SHEARER, C. 1920 Jour. Hyg., **18**, 337.

WINSLOW, C.-E. A., AND DOLLOFF, A. F. 1928 Jour. Bact., **16**, 67.

WINSLOW, C.-E. A., AND FALK, I. S. 1918 Proc. Soc. Exp. Biol. and Med., **16**, 67.

WINSLOW, C.-E. A., AND FALK, I. S. 1923a Jour. Bact., **8**, 215.

WINSLOW, C.-E. A., AND FALK, I. S. 1923b Jour. Bact., **8**, 237.

WINSLOW, C.-E. A., AND HOTCHKISS, M. 1922 Proc. Soc. Exp. Biol. and Med., **19**, 314.

A STATISTICAL INQUIRY INTO METHODS FOR ESTIMATING NUMBERS OF RHIZOBIA[1]

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INTRODUCTION

The importance of accurate methods for the estimation of bacterial numbers is apparent to any worker in bacteriology. The successful application of this science to practical problems was made possible only after development of methods which enabled census-taking of the bacterial population. The dairy industry, food products, sewage disposal, and water supply furnish examples of bacterial control placed on a firm foundation through the development and application of suitable methods for the enumeration of microörganisms. Of equal importance are counting methods to the research student engaged in more theoretical problems. Interpretation of data concerned with problems in metabolism and physiology of organisms can undoubtedly be made with greater assurance if the mass of cells which took part in the transformations is accurately known.

Most investigations are made with organisms whose characteristics are well known and counts made by either the plate or the direct methods are accepted without question. Such a procedure is probably not objectionable since the methods used have been thoroughly tested by numerous workers and found to be adequate and entirely trustworthy in the hands of a careful technician. However, certain organisms possess peculiarities in reproduction which tend to introduce errors in the ordinary counting methods

unless especial care is taken to eliminate these. In such cases it is advantageous to inquire into the accuracy of the methods employed. Examples of this latter type are the members of the genus *Rhizobium*, or root nodule bacteria. Due to their high gum production, which renders difficult proper distribution in dilutions, as well as to other little understood characteristics, e.g., the life cycle, attempts to enumerate these organisms by the plate count often give highly erratic results. Werkman (1927), comments, "Plate counts were abandoned since any strain of *Azotobacter* or *Rhizobium* forms some gum and plate counts were found unreliable." His lack of faith in the results of the plate count is no doubt shared by numerous other workers. The necessity of determining both viable and total counts in connection with certain research problems in this laboratory led to an investigation of methods that are commonly used to enumerate these bacteria. The purpose of this work was to establish the accuracy of the methods now employed and was not an attempt to develop a more reliable procedure. A problem of this nature is essentially one involving the origin of errors or deviations. In general, these can be classified as follows: (1) Those arising from non-uniformity or carelessness in technique; (2) those arising from failure to provide optimum conditions for the growth of the organism; (3) those due to chance. In a viable count the first two sources of errors are minimized in so far as possible by choice of medium, standardization of procedure, etc. In the case of direct counts only the first and third operate. Hence, if counts are made under carefully controlled conditions so as to eliminate sources 1 and 2 the deviations noted should be only those due to chance. If we compare the deviations or errors noted with those expected from chance alone and find that the expectancy is exceeded, we must conclude that our technique does not provide the proper conditions for results of sufficient accuracy. If the difference between errors noted and those due to chance is very large, the sources of errors should be eliminated if possible, or failing in this, the method should be used with full cognizance of the errors involved.

EXPERIMENTAL

The plate count

Method.   The estimation of bacteria by a plate count is the usual method employed in attempting to count viable bacteria. This method is based on the assumption that every living cell or aggregate of cells is capable of producing a colony if placed in a "suitable medium."   However, it is not always possible to elaborate a medium that will satisfy the assumption.   The depth of agar, choice of substrate, and the more complex effects of association tend to introduce errors in this method.   Under properly controlled conditions and especially with a pure culture, these errors are fairly constant, so that the method is adequate for comparable counts.   It must be realized, however, that every living cell is not capable of reproduction; consequently the count really represents cells or aggregates that reproduce and not total viable organisms.   Rubner (1904), in his work on the energetics of alcohol fermentation observed that the ability of yeast to metabolize and to form colonies on a malt agar plate were quite distinct. He says "Lebensfähigkeit und Wachstumsfähigkeit sind zwei Dinge die man auseinanderhalten muss."

Choice of substrate is a delicate problem that often is decided by personal likes and dislikes.   In this laboratory it has been found that Fred and Waksman's medium 79 (1928) gives the most constant counts with *Rhizobium* and it was therefore employed in this work:

Agar..	15	grams
Mannitol	10	grams
K_2HPO_4	0 5	gram
$MgSO_4 \cdot 7H_2O$.............................	0 2	gram
NaCl...	0.1	gram
Yeast water..	100	cc.
Water...	900	cc.

The yeast water is prepared by steaming 100 grams of fresh yeast (starch free) with 900 cc. of tap water for three hours, allowing to settle for several days, then centrifuging to remove débris. It contains about 1 mgm. of nitrogen per cubic centimeter.   Other

workers have used soil-extract agar, plant-extracts agar, etc., with success but we have not been able to find these media advantageous in our work. This is not intended as a criticism of these media, since success with a more or less suitable medium depends to a great extent on familiarity with methods of preparation and experience in duplication.

In each experiment a sufficient quantity of the above medium was prepared to last throughout the test. Ten cubic centimeters were measured with a pipette into tubes, sterilized, and these tubes melted by placing in a steamer for 15 minutes before pouring the plates each day. The plates were chosen for uniformity of size so that the same depth of agar was obtained in each plate. This point is important in counting the *Rhizobia* because of their peculiar sensitiveness to changes in oxygen tension relations. Another factor which must be carefully controlled is the temperature of the agar at pouring since the organisms are very sensitive to heat, Alicante (1926). A special water bath was used which kept the tubes of agar to be poured at $42° \pm 1°C$.

The water blanks were accurately measured in 9 and 99 cc. portions. The former were placed in test-tubes and the latter in 6 oz. bottles so as to avoid undue losses in sterilization. Ordinarily tap water was used in the blanks but in a few experiments physiological salt solution was used. Accurate 1 cc. Mohr pipettes were used for all dilutions and these were made at room temperature, about 25°C. In order to insure distribution of the organism, sterile glass beads were placed in the flask used for growing the organism and the flask shaken 25 times before a sample was withdrawn for counting. The first dilution bottle was also provided with sterile beads for breaking up aggregates of bacteria. All dilutions were shaken 25 times before a sample was withdrawn.

Statistical treatment. If a standard technique made all errors other than those arising from chance constant, the method would be satisfactory since usually the counts are wanted for comparative results; absolute numbers, while desirable, are not actually essential. Fisher, Thornton, and MacKenzie (1922) and Fisher (1930) have shown that the conditions laid down in the Poissan Exponential Summation are fulfilled in a plate count made under

ideal conditions. These conditions are (1) the probability (p) that a given organism from a large population falls in a certain plate shall be very small; (2) the number (n) of organisms exposed to this probability shall be very large. With similar assumptions Poissan showed that the probability that the number of occurrences shall be x is given by the expression $e^{-m}\dfrac{m^x}{x!}$. x is always a whole number (as the colonies per plates), while m is the mean value of x (average of all plates). For large values of m, the distribution is essentially normal with a standard deviation equal to $\sqrt{m}$. Now in order to test whether the variations in plate counts are due to those arising from random sampling it would be necessary to have a large number of plates, say 100, of each dilution and to compare the distribution of the number of colonies per plate with that predicted by the Poissan law. In actual practice we have only 3 to 5 plates of each dilution, hence such a comparison would be useless, due to the small number of samples. However, Fisher and associates have shown that for small samples of a Poissan series an index of dispersion (measure of variation) can be calculated from the sum of the squares of the deviations from the mean, divided by the mean. This statistic is called Chi square and expressed mathematically it is

$$X^2 = \frac{\Sigma\,(x - \bar{x})^{2*}}{\bar{x}}$$

* Mathematically this is equivalent to Pearson's "Goodness of Fit" test.

where x is the individual value observed, e.g., colonies per plate, $\bar{x}$ is the mean value (average of all plates, and Σ denotes summation. The advantage of this statistic over others that might be used, e.g., standard deviation, variance, etc., is that its distribution is independent of the mean value ($\bar{x}$); hence a number of small samples from a series of parallel platings can be used to test whether these samples are taken from the theoretical Poissan distributions. This is made possible through the fact that the values of X^2 calculated as above will be distributed in a known manner if the samples are from a Poissan series. Tables by El-

derton (1902) give the number of times X^2 will exceed successive integral values for values of n from 0 to 30, where n is one less than the number of parallel plates. Fisher, *et al.* (1922), have shown that soil counts made under standardized conditions have a distribution of X^2 which is very close to the theoretical one and confirm the view that the bacterial counts on the parallel plates vary in the same way as small samples from Poissan Series. In some cases the distribution of X^2 departed from the theoretical but on investigation it was found that (1) occurrence of certain organisms which reduced colony development or (2) deficiencies in the medium, gave rise to (1) excessive and (2) subnormal variation in parallel plates. This led to the observed abnormal departure from the theoretical in the distribution of X^2. The authors conclude, "Any significant departure from the theoretical distribution is a sign that the mean may be wholly unreliable."

Experiment I. Three-plate data. In view of the analysis of Fisher, Thornton, and MacKenzie of different sets of data concerned with counts of variable bacterial flora, it would be expected that the distribution of X^2 from counts made on a pure culture should show a very good agreement with the theoretical distribution. In this case the selection of media to suit the organism is simplified and errors arising from antagonistic effects of one type of organism on another are eliminated.

In the course of experiments dealing with the growth of *Rh. trifolii* on different sources of nitrogen it was noticed that parallel plates (two) often showed wide variation so that the mean was highly untrustworthy. In order to secure more reliable data it was decided to standardize the technique as much as possible and to make three parallel plates instead of two. In work of this kind there is a limit to the number of parallel plates that can be made because of the time factor involved. In a laboratory test in which a comparison of three or four types of treatment are being investigated, e.g., effect of the sources of nitrogen on the growth of an organism, it would not be feasible to make parallel platings of five or more since the time involved would affect the counts to such an extent that the variability would not be decreased by the added number of plates. In order that the find-

ings of Fisher *et al.*, might be applied to our actual laboratory practice it was decided that three or four plate data should be examined.   After preliminary experiments concerned with composition of medium, water blanks, temperature, etc. the technique described under *Methods* was adopted.   The plates were incubated at 28°C. and counted after 5 and 10 days but only the 10-day count is considered here.

Following the standardization of the technique, data for about 100 sets of 3 plates were gathered.   Upon calculation of the X^2's

TABLE 1

Comparison of observed distribution of X^2 with theoretical for three plates

X^{2*}	THEORETICAL m	OBSERVED x	$x - m$	$\dfrac{(x - m)^2}{m}$
0.00 –0.438	12	12	0	
0.438–0.973	12	11	−1	0 083
0.973–1 663	12	7	−5	2 083
1.663–2.636	12	13	1	0 083
2 636–4.300	12	14	2	0 333
4 300–	12	15	3	0 750
Totals	72	72		$X^2 = 3.332$†
				$P = 0.65$

*These values of X^2 represent the distribution of this statistic in the data examined.

† This X^2 is the total of $\dfrac{(x - m)^2}{m}$ for all cells and is the value used to test the goodness of fit of the observed data with the theoretical.   See Fischer (1922, 1930).

for these data, it was found that there was a great excess of large values of this statistic, in other words, the data showed an excessive variance.   Upon investigation of these large values of X^2's it was found that most of them arose from counts of the following type: 159, 162, 103; 174, 160, 107; 73, 69, 54.   It will be noticed that two of the plates do not show unduly wide variation, but the third plate is so much smaller than the other two that the variance and hence X^2 becomes very large.   These cases caused a fairly large number of X^2 figures to have values from 10 to 30.   Now in 100 samples with $n = 3$, there should be only one sample

with X^2 greater than 10. Accordingly the data were examined and those sets eliminated which showed a value of X^2 greater than 10 and *in which this value was due to the large deviation of one plate*. The distribution of X^2 from the data after this elimination is shown in table 1. The test of agreement was made by the Pearson test of *Goodness of Fit* (Fisher, 1930) and the result shows that 65 times out of a 100, one would expect a worse fit due to chance alone—a very good agreement.

Experiment II. Four-plate data. From the results with the 3-plate data, there were indications that the erratic results noted with two parallel plates were due to factors that sometimes caused

TABLE 2

Comparison of observed distribution of X^2 with theoretical for four plates

X^2	THEORETICAL m	OBSERVED x	$x - m$	$\dfrac{(x-m)^2}{m}$
0.5	8 94	6	−2.94	0 97
1.5	10 29	14	3 71	1.34
2 5	8.14	8	−0 14	0.02
3.5	5 85	3	−2 85	1.39
4 5	4 05	6	1 95	0 94
6.5	4 50	4	−0.50	0.06
>6.5	3.23	4	0.77	0 18
Totals........	45	45	$X^2 = 4.90$	
			$P = 0.56$	

a certain plate to show an abnormal deviation from the mean of the population. With two plates it is impossible to judge the erratic plate; with three, one might select that plate which departs most markedly from the average of the other two. However, this is somewhat arbitrary and might give rise to bias if a large number of plates in a given series are unusually variable. In order to determine if these erratic plates were the exception rather than the rule, a series of 5 plates were made, then one plate eliminated from each count so that a 4-plate series resulted. If there was any one plate in the 5 replicates that showed marked deviation from the others this was eliminated, otherwise the elimination was left to chance by throwing out the third plate counted. It

was necessary to eliminate by choice in 18 of the 45 sets and as noted in experiment I, the majority (14) were plates that gave a much smaller count than the other four parallels.   The distribution of X^2 for this set of data is given in table 2.   In table 1 the distribution was made in sextiles, i.e., the expected number in each cell is the same.   In table 2 the distribution of X^2 is given in integral values grouping the values at the extremes so that the expected number in each cell is three or more.   While 45 samples
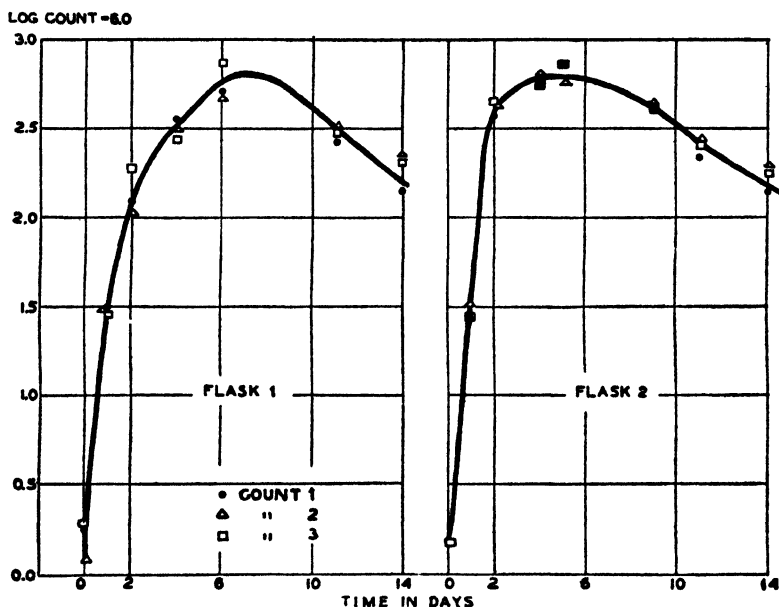
CHART 1. GROWTH CURVES OF *Rhizobium trifolii* IN MEDIUM 79

are hardly sufficient to make a through test of the distribution, the probability found (0.56) that the deviation noted arose from sampling is indicative that the distribution of values tends toward the expected ones.

The data discussed above were counts made periodically on the growth of *Rhizobium trifolii*, Culture 200, in Medium 79.   Chart 1 shows two of the growth curves made from the counts.   In this particular experiment duplicate flasks were taken and 3 counts made on each flask after varying number of days.   These counts

were made with three parallel plates and in the case of those sets of three that had one plate markedly different from the other two the average of the two plates that showed the least variation was taken as the correct count. The distance of the average of the individual counts from the "best" line is a measure of the variability among the triplicate counts. These growth curves are quite similar to those found for other organisms, exhibiting a

TABLE 3

Comparison of counts of Rhizobium trifolii culture 209 in replicate flasks

	AGE						
	0 day	1 day	2 days	3 days	5 days	7 days	10 days
Experiment I. Three plates							
Flask 1......................	0.31*	19 0	170	253	257		
Flask 2......................	0.21	14.7	125	239	227		
Experiment II. Three plates							
Flask 1......................	0.66	19.3	173	490	463		
Flask 2......................	0 38	46.0	309	508	464		
Experiment III. Four plates							
Flask 1......................	7.2	23.3	297	337	229 (?)	335	280
Flask 2......................	1.7	4.8	78	118	160	187	188
Flask 3......................	2.4	11 0	128	148	349	267	271
Flask 4	0.8	68.3		229	450	495	458

* All counts expressed as millions per cubic centimeter.

period of logarithmic increase during the first two days, a phase of negative growth acceleration during the second to fourth days, a brief period of maximum growth followed by a period of "death" that approaches the logarithmic. The growth curves in the two flasks were quite similar in this experiment but this is not always the case as shown in table 3. In a comparison of growth on different media this variability of replicate flasks would have to be taken into consideration in interpretation of the data.

The direct count

Method. The direct count of bacteria by means of a haemocytometer was first made in 1898 when Winterberg (1898) made use of the apparatus devised by Lyons and Thoma for estimation of blood corpuscles. The Petroff-Hausser counter has been developed especially for the counting of bacteria by this method; its chief advantage is the decreased depth of the chamber (0.02 mm.) so that it is not necessary to move the microscope up and down in order to locate the bacteria in different levels of the counting chamber. The direct count offers many obvious advantages over plate counts if knowledge of the total number of bacteria present rather than viable bacteria will suffice. In many experiments this information is that desired and a direct count is of much more value than a plate count. However, it has the disadvantage that each bacterium per square on the Petroff-Hausser counter represents 20,000,000 organisms per cubic centimeter of the sample and therefore can be used to advantage only when the number of organisms present is very high. In the experiments reported below, most of the counts were made with the dark field. Some were made with the light field in which case the 1 cc. of the diluted culture was mixed with 1 or 2 cc. of Meissner solution. The dark field work is preferred as the counting is easier on the eyes and it is less difficult to distinguish the bacteria from occasional débris. The counts were made on 48 to 120 hours cultures in liquid medium, with and without carbohydrate, and on suspensions of the organism from agar slants. In the latter case the growth was washed with sterile water into 100 cc. volumetric flasks, then further diluted according to the numbers present. Beads were used in all cases to break up clumps.

Statistical treatment. "Student" (1907) from theoretical consideration of the count of yeast cells in a haemocytometer arrived at a distribution of the counts per unit area that followed the Poissan Exponential Summation. He showed that under favorable conditions this theoretical distribution was actually realized in practice. He further pointed out that the standard deviation of the count was $\sqrt{m}$; hence the accuracy of any count was pro-

portional to the total number counted. "Student's" counts were made with the purpose of verifying Poissan's law rather than to test the accuracy of the count and his technique was not exactly

TABLE 4

Comparison of theoretical distribution with observed when counting Rhizobium trifolii in Petroff-Hausser counter

NUMBER PER SQUARE	THEORETICAL m	OBSERVED x	$x - m$	$\dfrac{(x - m)^2}{m}$	$\dfrac{(x - m)^2}{m}$ (GROUPING)
I. Mean—5.16					
<2	14 28	16	+1 72	0 21	0.21
2	30 77	25	−5.77	1 08	0.88
3	52.81	50	−2 81	0.15	
4	67 98	73	+5 02	0.37	0.71
5	70 01	75	+4 99	0 36	
6	60.10	57	−3.10	0 16	0 16
7	44 22	49	+4 78	0 52	0.52
8	26.46	23	−3 46	0 45	0.45
9	16 30	18	+1 70	0 18	0 18
10	8 40	5	−3 40	1 37	1 37
>10	6 65	9	+2.35	0 83	0.83 ·
Totals......	400	400	$X^2 = 5\ 68$ $P = 0.77$		$X^2 = 5.31$ $P = 0.62$
II. Mean—2.50					
0	32 83	34	+1.17	0.04	0 04
1	82 08	68	−14 08	2.41	2.41
2	102.60	112	+9.60	0 90	
3	85 50	94	+8 50	0.84	1.60
4	53.44	55	+1 56	0.05	
5	26.72	21	−5 72	1.22	1.22
6	11.12	12	+0.88	0.07	0 07
>6	5.62	4	−1 67	0.49	0 49
Totals......	400	400	$X^2 = 6.02$ $P = 0\ 43$		$X^2 = 4\ 83$ $P = 0\ 30$

that followed in actual laboratory practice. For example, he fixed the yeast cells in gelatin under the cover slip so as to prevent movement of the cells during the counting. Since under the

more or less ideal conditions obtained by "Student," it was shown that the distribution of organisms per unit area followed Poissan's law, a good test of the accuracy of a laboratory count can be made by comparison of the theoretical distribution given by Poissan's Exponential Summation with that actually obtained. This was done on a large number of cultures of rhizobia. Due to gum formation, clumping might be expected to interfere with the accuracy of the count, but in actual counting it was found that clumps were not frequent and were easily identified by their large size. In making the counts an effort was made to estimate the number of organisms in each clump and, if a sample showed a large number of these, to discard the count. The observed distributions were compared with the theoretical by means of Pearson's *Goodness of Fit* test. The table given by Fisher (1930) was used to determine P, *i.e.*, the probability of a worse fit through chance; in this table n is two less than the total number of the various frequencies compared. For example in table 4, Part I, eleven frequencies are compared in the ungrouped data hence the table is entered with $n = 9$.

Experiment I. Six counts were made on *Rhizobium trifolii*, culture 209, in which the entire 400 small squares were counted and the distribution of organisms per square compared with the theoretical value given by the expansion:

$$Ne^{-m}\left(1 + m + \frac{m^2}{2!} + \frac{m^3}{3!}\cdots\right)$$

where N equals total squares counted and m equals mean number of organism per square. The table of Soper (1914) was used to calculate the theoretical distribution. Table 4 gives results of two typical counts. The probabilities that the deviations noted arose from chance, 0.77 and 0.43, indicate that the observed distributions followed the theoretical Poissan law, hence the accuracy of the counts made under these conditions can be immediately determined since the standard deviation of the mean of this distribution is equal to $\sqrt{\frac{m}{N}}$. Thus in Part I, the mean number of organisms per square was 5.16. This has a standard deviation of

$\sqrt{\dfrac{5.16}{400}} = 0.1135.$ If we assume that the true mean is within plus or minus three times the standard deviation of the observed mean, we know that the true mean is between the limits 4.82 and 5.60 and the maximum error in the observed mean is 6.6 per cent.
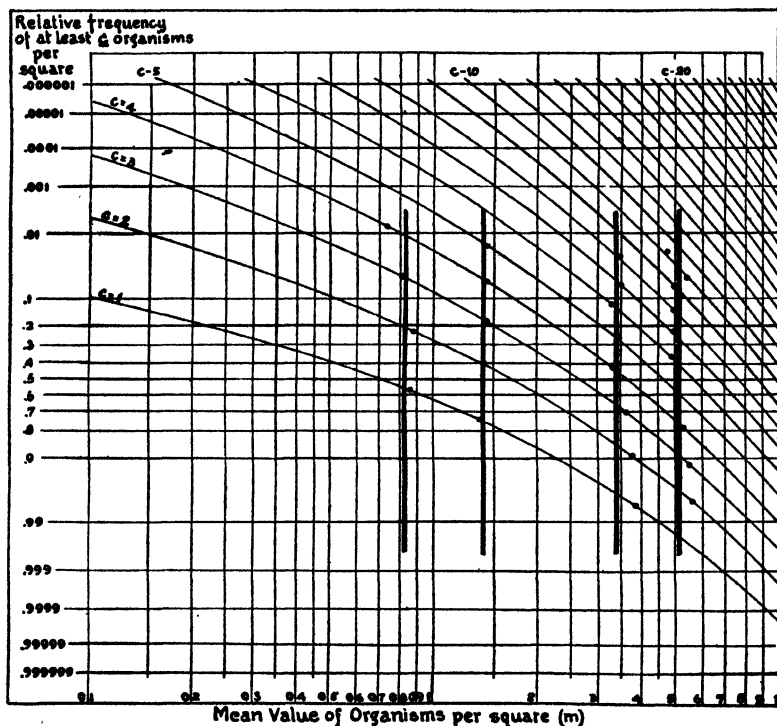
CHART 2. COMPARISON OF OBSERVED DISTRIBUTIONS OF *Rhizobium trifolii* WITH THEORETICAL GIVEN BY POISSAN EXPONENTIAL SUMMATION

Even when grouped in an unfavorable manner by placing together consecutive positive and negative residuals (x-m) the probabilities are such as would be expected from perfectly normal data. Thorndyke (1926) has shown that if the *relative frequency* of obtaining at least c occurrences in data from a Poissan distribution is plotted against c on a special graph paper[2] which she describes,

[2] Arithmetic Probability Paper No. 3127.   Designed by Hazen, Whipple and Fuller.   Codex Book Company, Inc., New York.

the points should follow a straight line drawn from the number on the base which gives the average number of occurrences (m). Thus, in order to test any data to determine whether they were drawn from a population with a Poissan distribution it is only necessary to plot the relative frequency of at least c occurrences against c and observe whether the points so located follow the proper straight line.   For details of the construction of this graph paper and the reasons why these points must lie on a straight line for a Poissan distribution, the reader is referred to the original article.   Data from four more counts of 400 squares were tested in this way as shown in chart 2.   The fit to the straight line is satisfactory in every case, especially the points in the center where the data are more reliable.

These results indicate that under laboratory conditions counts made with the Petroff-Hausser bacteria counter followed the theoretical distribution satisfactorily and therefore confirmed the accuracy and reliability of counts obtained by this method.

Experiment II.   In actual practice it is not feasible to count the entire 400 squares as the added accuracy obtained would not repay for the time required.   In subsequent work counts were made of 144 small squares (9 large ones) distributed symetrically over the total area of 400 mm.   Dilutions were made so that the average number per small square was between 4 and 5.   This average number was found to lead to distributions most readily counted and since a total of about 600 organisms are counted the result is probably accurate within 8 per cent which is sufficient for most routine work.   Fifty tests counting 144 squares were made and the observed distributions compared with the theoretical ones.   The organisms used were *Rhizobium trifolii*, *Rhizobium meliloti*, *Rhizobium japonicum*, and *Rhizobium leguminosarum*. These were grown in liquid medium with and without added carbohydrate, on agar slants and on agar in 16 oz. bottles.   The probabilities found that the deviations noted arose from random sampling were grouped as follows:

15 samples had probabilities between 0.00 and 0.20
6 samples had probabilities between 0.20 and 0.40
10 samples had probabilities between 0.40 and 0.60

10 samples had probabilities between 0.60 and 0.80
9 samples had probabilities between 0.80 and 1.00

Since the expectation in each group is 10 it can readily be seen that the agreement with theory of the 50 distributions examined

TABLE 5

Effect of dilution

	DILUTION	COUNT	$\frac{x - \bar{x}}{S.D.} = t^*$
		$\times 10^{-6}$	
Culture 209	1:1	121	1.42
		144	3 66
	1:2	89	1.68
		101	0.51
	1:3	80	2.55
		106	0.03
	1:4	103	0 32
Mean		106 3	
Standard deviation...............		10 3	
Culture 29:16	1:20	270	3.32
		312	1.01
	1:10	374	2.39
		366	1.95
Mean.............		330.5	
Standard deviation		18 20	
Culture 29:16..........................	1:8	550	0.55
		528	0.40
	1:10	513	1.05
		558	0.89
Mean		537.3	
Standard deviation................................		23.18	

* x = observed count.
$\bar{x}$ = mean of counts.

is fairly close to that expected. If the observed probabilities are grouped in 4 series the agreement with expectation is even closer. This agreement was confirmed by summing the X^2's and the n's used in making the individual tests for goodness of fit. Fisher

(1930) shows that in this case if $\sqrt{2x^2} - \sqrt{2n-1}$ is less than 2.00 the data may be considered normal. For the 50 counts made, this difference was $+0.57$; hence the data are entirely satisfactory. It can be concluded on the basis of these 50 counts that, even in routine work, the theoretical distributions are achieved closely enough to establish confidence in the accuracy of the counts made.

Experiment III. The effect of dilution and the checks to be expected on duplicate counts were next investigated. Three cultures were taken and duplicate counts made at different dilutions; the mean of all the counts and the standard deviation were calculated. The statistic, t = deviation from mean of individual counts divided by the standard deviation was then determined. If this is less than 3.0 the counts may be considered to be unaffected by dilution. Table 5 shows that only 2 out of 15 tested indicated abnormal variation in count due to dilution.

The checks on duplicate samples were determined in a similar manner. Twelve sets of duplicates and two sets of triplicate counts were examined and the value of t was determined. Of the 18 values of t so calculated, 9 were less than 1.00; 5 were between 1.00 and 1.50; and 4 were between 1.50 and 2.00; one was 3.04.

Other methods of counting

A few experiments were made in which direct counts were obtained by the Fries method (1921). However, this method did not prove satisfactory since the counts obtained were erratic and in a large number of cases they were less than the corresponding plate count, an entirely unexpected result. Winslow (1905) reports a similar finding; he found a disappearance of 100,000,000 bacteria per cubic centimeter within four hours, which he concluded to be due to loss of staining property. With rhizobia another possibility may be suggested. Examination of slides reveals rod-shaped bacteria with rounded granular bodies staining heavily. Always in the same fields are numerous spherical bodies the size of those contained within the rods, staining similarly. At the time of examination, there was considerable doubt as to whether or not these granular bodies should be included in the count; but

owing to extreme difficulties in drawing lines of distinction between them and precipitates of dyes and medium and dirt particles on the slide, it was deemed best not to include them. That these granular bodies may have been released from the banded cells during the course of vigorous shaking, has been suggested as a possible explanation for their presence on the slides; it may also be an explanation for the increased plate counts over the Fries counts, if we may assume that the granules are cell fragments or stages in the life cycle capable of reproduction.

The dilution method for estimation of viable bacteria was also tried. One cubic centimeter of each dilution was added to 5 tubes of litmus milk and to 5 tubes of Medium 79. Readings of the tubes were made after 10 days and the most probable number of organisms determined from McCrady's tables (1918). There was little difficulty in determining growth in either the litmus or the yeast-water mannitol solution but the results obtained indicated that some factor was present that interferred with the accuracy of the counts. For example most of the significant figures were of the type *500*. It would appear that the fundamental assumption of this method, i.e., that one cell can initiate growth is not always fulfilled with this organism. It is more likely that fairly large numbers of organisms must be present in order that growth can start in which case all the tubes are positive, but as soon as the number of organisms become smaller than some critical value, growth is not initiated. Such a condition would interfere with the variations in growth due only to a chance distribution which is the basis of the dilution method. The work of Allyn and Baldwin (1930) on the dependence of growth on the oxidation-reduction character of substrate lends support to the view that initiation of growth may be a function of population rather than of individual cells. This may also be a factor in the case of erratic plate counts.

SUMMARY

Plate counts of members of the genus *Rhizobium* are very erratic unless especial care is taken to maintain a standardized technique. Even if the latter is made uniform, the variability among individ-

ual plates is often larger than that attributable to random sampling. In such cases the abnormal variability usually results from one plate in a series, rather than from uniform variation among the replicates.

This variability can be partially eliminated by an increase in the number of parallel plates. Also, if three or more plates are made in parallel, the elimination of any one plate that is markedly different from its replicates can be made without bias. Sets of three and four plate data examined after elimination of any plate that was decidedly at variance with its replicates indicated that the variations noted in the remaining plates could have arisen by chance alone.

The cause of this abnormal variation was not investigated but other experiments and observations indicated that (1) stages in the life cycle and (2) unique growth-initiation requirements of the individual cells or stages, especially in regard to the oxidation-reduction character of the medium, may be factors involved.

In any study requiring a viable count the use of as many parallel plates as possible without introducing an undue time factor is desirable. In all cases at least three replicates are advisable.

Direct counts of rhizobia by means of a Petroff-Hausser counting chamber can be made under laboratory conditions in such a manner that the variance is that due to chance alone. In this case the distribution of the cells per unit area follows the Poissan Exponential Summation, and the accuracy of the count will be dependent solely on the total number of organism counted. The standard deviation of such a count is equal to $\sqrt{m}$ and the standard deviation of the mean $= \sqrt{\dfrac{m}{N}}$ where m is the mean number of organism per unit area, and N is the total squares counted.

The direct count with the Petroff-Hausser or similar bacteria counting chambers proved to be the most accurate of all the methods investigated and its use whenever possible is advised.

REFERENCES

ALICANTE, M. M.   1926   Soil Sci., **21**, 93.

ALLYN, W. P., AND BALDWIN, I. L.   1930   Jour. Bact., **20**, 417.

ELDERTON, P.   1902   Biometrika, I, 155.

FISHER, R. A.   1930   Statistical Methods for Research Workers, Third Ed., London.

FISHER, R. A., THORNTON, H. G., AND MACKENZIE, W. A.   1922   Ann. Appl. Biol., **9**, 325.

FRED, E. B., AND WAKSMAN, S. A.   1928   Lab. Manual of General Microbiology, New York.

FRIES, K. A.   1921   Centralbl. f. Bakt., I Abt., **86**, 90.

McCRADY, M. A.   1918   Pub. Health Jour. (Toronto), **9**, 201.

RUBNER, M.   1904   Arch. Hyg., **49**, 355.

SOPER, H. E.   1914   Biometrika, **10**, 25.

"STUDENT."   1907   Biometrika, **5**, 351.

THORNDYKE, F.   1926   Bell System Tech. Jour., **5**, 604.

WERKMAN, C. H.   1927   Jour. Bact., **14**, 335.

WINSLOW, C.-E. A.   1905   Jour. Inf. Dis. Suppl., **1**, 209.

WINTERBERG, H.   1898   Zeitschr. Hyg., **29**, 75.

ON THE DECOMPOSITION OF AGAR-AGAR BY A N AEROBIC BACTERIUM[1]

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A study of the processes of decomposition of agar-agar by microörganisms involves first of all a knowledge of the chemical nature of the agar. This material is prepared from certain marine algae and, due to the fact that it is used as a food in the Orient and for medicinal purposes in Europe and America, as well as due to its employment for the preparation of culture media, its chemical nature has received considerable attention.

It has been found that the composition of the agar varies with the nature of the plant from which it is obtained. Japanese agar is usually prepared from *Gelidium corneum*, while other species of *Gelidium* and species of *Gracillaria, Fucus* and *Eucheuma* are also employed in different parts of the world.

Although agar contains a considerable amount of ash, the major part of it consists of one or more hemicelluloses, or carbohydrates which are readily hydrolized by hot dilute mineral acids. The most important constituent of the agar is a galactan, first referred to by Payen as gelose, to which he gave the formula $C_6H_{10}O_5$. Czapek (1913) in summarizing the results of previous investigators, concludes that one-third of the agar consists of galactan. On treatment of agar with nitric acid, mucic and oxalic acids are produced. Pentosans are usually reported to be present in agar but in very small amounts. Fellers (1916) reported the presence

of 3.12 per cent pentosan and 22.87 per cent galactan in purified (Bacto) agar. The galactan in the agar is usually referred to as δ-galactan.

The inorganic constituents are made up chiefly of Ca and Mg sulfates. Without going into a detailed discussion of the voluminous literature on the chemical composition and uses of agar (Fellers, 1916), a summary is given in table 1 of a few recent analyses of this preparation. Attention is directed to the fact that the composition of the agar varies, depending upon the plant

TABLE 1

Chemical composition of agar

Per cent of air-dry material

Moisture	15.29*	16.57†
Ash	4.23	3 85
S	1.77	2 65
Ca	0 66	0 92
Mg	0 48	0 57
Na	0 11	0.25
K	0.11	0.07
Cl	0.03	0 22
P	0 02	0.05
N	0 30	0.37
Fat	0.37	0.30
Fiber	0 89	0 80
Carbohydrates (N-free extract)	77.34	76.15

* Forbes et al. (1913).
† Fellers (1916). Average of 15 samples; ash constituents reported as oxides.

from which it was obtained, and upon methods of preparation and purification.

Haas (1921) has shown that the ash content of carrageen obtained from *Chondrus crispus* cannot be reduced by dialysis below 14.6 per cent; the ash consists principally of $CaSO_4$. In solutions of original carrageen, Ca-ions are present, but not sulfate ions. Haas concluded, therefore, that the calcium and sulfate ions are integral parts of the carrageen molecule and are present as a sulfuric ester:

$$R \underset{O \cdot SO_2 \cdot O}{\overset{O \cdot SO_2 \cdot O}{<\quad>}} Ca$$
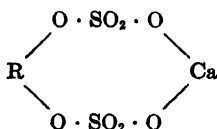
Neuberg and Ohle (1921) found that the sulfur present in the ash of agar-agar was less than one-half of the total sulfur in the original agar. Samec and Ssajevic (1922) suggested that the formula for agar was $(C_6H_{10}O_5)_{54}SO_4H$. Fairbrother and Martin (1923) concluded that agar-agar consists principally of the calcium salt of an acid sulfuric ester, namely $(R \cdot O \cdot SO_2 \cdot O)_2Ca$.

Several bacteria capable of liquefying agar have been isolated in the past. Here belong the *Bac. gelaticus* of Gran (1902), the *Bact. betae-viscosum* of Panek (1905), the *Bac. Nenckii* of Biernacki (1911), *Microspira agar-liquefaciens* of Gray and Chalmers (1924), *Vibrio Andoi* of Aoi (1925), Aoi and Orikura (1928), and others. Only one of these bacteria was isolated from sea water. The others were isoated from soil and other substrates. The specific bacterium is very abundant in sea waters, but has been least studied.

Gran found that *Bac. gelaticus* produced an enzyme which could hydrolize agar to sugar. *Vibrio agar-liquefaciens* decomposed cellulose in addition to agar. The organism isolated by Aoi and Orikura (1928) readily decomposed xylan, mannan and starch, but did not decompose cellulose. This organism was isolated by Aoi (1925) from manure. The bacterium did not develop on nutrient agar, but grew well on an inorganic medium containing agar as a source of energy; it made good growth on Gran's medium, when 0.1 per cent NaCl was substituted for 3 per cent; it also grew well with mannan as a source of energy. The organism was an obligate aerobe and was killed on heating at 45°C. for ten minutes.

H. and E. Pringsheim (1910) employed agar-agar as a source of energy for nitrogen-fixation. They found that in the decomposition of agar by *Bac. gelaticus*, there are formed substances which reduce Fehling's solution and which are used by the nitrogen fixing bacteria as sources of energy. However, Lundestad (1938), who recently isolated an agar-destroying organism from sea water, could not demonstrate any reducing sugars in the process of agar liquefaction. The quantitative destruction of the agar has so far not been studied.

Occurrence and nature of organism used

In the course of a study of the microflora of marine sediments, an organism capable of energetic decomposition of agar, as indicated by the extent of liquefaction of an agar medium, was isolated. Hundreds of thousands of cells of this organism were found per gram of the mud sediment of Williams Island (Bahama Islands), especially in the upper regions of the mangrove-swamps, but also in the calcium carbonate sediment around the coast. The abundance of the organism could be very readily determined by merely counting the number of depressions on the agar plate produced by the specific organisms.

The organisms developed best on the lactate-agar of Drew, (1915), this medium having the following composition:

		grams
Sea water		1,000
KNO₃		0 5
Na₂HPO₄		0 25
Calcium lactate		2 00
Agar		18 00

The organism was isolated on a medium containing 0.5 gram K_2HPO_4 and 12.5 grams of agar in 1000 cc. of sea water. It was best cultivated upon a medium containing 0.2 gram peptone and 10 grams agar in 1000 cc. of sea water. It grew, however, well upon sea water agar with KNO_3, $(NH_4)_2SO_4$ or asparagin as sources of nitrogen.

Colonies. Almost colorless and transparent, with yellowish-white surface. The bacterial slime is yellowish.

Morphology of organism. Small, motile rod, non-spore forming, 2 to 4 by 0.5 to 0.7μ. Stains well with bacterial dyes.

Abundance. One gram of moist mud containing 50,000 to 200,000 cells, as determined by plate method.

The organism seems to be closely related to the *Bac. gelatinus* isolated by Gran (1902), possibly being identical with it.[2]

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[2] A detailed study of the morphology of this organism and its relation to the other agar-liquefying bacteria will be published later by the Junior author in the Centralblatt für Bakteriologie (II Abteilung).

Decomposition of agar

For the following experiments the ordinary Difco Bacto Agar has been employed. This agar had the following composition:

	per cent
Moisture	15.6
Reducing sugar, on hydrolysis with 2 per cent HCl in flowing steam for 5 hours	64.8
Ash	2 3
Nitrogen	0.14

In the first experiment, 200 cc. portions of a medium containing 1.0 per cent agar in sea water plus $NaNO_3$ as a source of nitrogen (2 grams per liter) were placed in a series of 500 cc. Erlenmeyer flasks, sterilized, inoculated with 2 strains of the organism, and connected with the respiration apparatus, for absorbing the CO_2 given off in the process of decomposition. Table 2 shows the amounts of CO_2 given off, measured as carbon. Table 3 gives the chemical composition of the cultures as compared with the control, and brings out further the amount and nature of decomposition that had taken place.

The results presented in tables 2 and 3 show that considerable decomposition of the agar has taken place in both cultures. Nearly a third to a fourth of the agar was liquefied in 31 days. Culture 2 was somewhat more active than culture 1. If the analysis of the liquefied portion alone were presented, considerably greater decomposition would have been brought out; probably over 80 per cent of the hemicellulose in the agar would have been found to have disappeared. However, since the amount of liquid available for analytical work was very small, and since the unliquefied agar has probably also undergone partial decomposition and considerable diffusion of the salts has no doubt taken place, the total culture was hydrolyzed with 2 per cent hydrochloric acid in flowing steam and submitted to analysis.

The two cultures brought about the decomposition of from 26 to over 37 per cent of the agar, as shown by the amount of hemicellulose that disappeared. On comparing the amount of hemicellulose decomposed (calculated as carbon content of the sugar found after hydrolysis) to the amount of carbon liberated as

carbon dioxide, it is found that 71 per cent of the carbon of the hemicellulose decomposed in the agar has been liberated as gas, while 29 per cent has either been left in the form of intermediary products or utilized by the organism for the synthesis of its cell substance. The fact that considerable synthesis has taken place is brought out by the consumption of the nitrate-nitrogen and its transformation into organic nitrogen.

TABLE 2

Evolution of CO_2 in the course of decomposition of agar by a bacterium

Milligrams of carbon from 200 cc. of culture

CULTURE	DAYS OF INCUBATION								TOTAL
	8	10	12	15	19	22	25	31	
Control				3.2				2.7	5 9
No. 1.....................		14 8		17.8	14 3	22 2	22 6	16 6	108 7
No. 2	19.3	17 0	11 1	12 6	17 2	26 9	16 3	27.9	148 3

TABLE 3

Nature of agar decomposition by a bacterium

CULTURE	REDUCING SUGAR ON HYDROLYSIS		NO$_3$-N IN THE AGAR		HEMICELLULOSE CARBON CONSUMED* CO$_2$ (AS CARBON) LIBERATED	HEMICELLULOSE (AS SUGAR) CONSUMED NITROGEN CONSUMED	C LIBERATED AS CO$_2$ NITROGEN CONSUMED
	Left	Decomposed	Left	Consumed			
	mgm	*mgm.*	*mgm.*	*mgm.*			
Control	1 307	0	60 5	0			
No. 1	964	343	38 9	21.6	1.39	15 9	5.03
No. 2	823	484	36 8	23 7	1 43	20 4	6 26

* Assuming that the carbon content of the hemicellulose is 44 per cent.

The fairly narrow ratio between the amount of hemicellulose in the agar decomposed and the nitrogen assimilated points further to the abundant synthesis of bacterial cells.

In order to throw further light upon the metabolism of this specific organism, it was grown in a synthetic solution with ammonium sulfate as a source of nitrogen and with glucose, starch and mannan as sources of energy. The media were sterilized, inoculated and incubated at 27°C. for 24 days. There was very little growth in all of these cultures; whether this was due

to the low salt concentration (plain distilled water being used in this experiment), or to the fact that ammonium salt was used as a source of nitrogen (this substance was found to be inferior to nitrate nitrogen for this organism), or to the inability of the organism to attack these sources of energy, still remains to be determined. The data presented in table 4 point, however, to some very interesting conclusions.

The results show that only about 10 per cent of the carbohydrates were consumed by the organism. However, large quantities of reducing sugars were produced from the starch and mannan. This brings out the fact that even when conditions are not very favorable for the growth of the organism, the latter

TABLE 4

Decomposition of different carbohydrates by an agar-liquefying bacterium

NATURE OF CARBOHYDRATES	AMOUNT OF SUGAR PRESENT		TOTAL SUGAR ON HYDROLYSIS WITH 2 PER CENT HCl SOLUTION		
	Control	Inoculated	Control	Inoculated	Sugar consumed
	mgm.	*mgm*	*mgm*	*mgm*	*mgm.*
Glucose	252	235	235	219	16
Starch......	0	111	212	186	26
Mannan	0	89 4	206	192	14

continues to produce an enzyme which is capable of hydrolyzing the polysaccharides to reducing sugar and, as a result of the fact that the organism itself is not able to consume this sugar immediately, the latter continues to accumulate. This may be the reason why it is so difficult to demonstrate the production of sugar in the process of cellulose decomposition by fungi and bacteria. When conditions are made unfavorable for the growth of the organism but favorable to enzyme action, the sugars will accumulate (Payen, 1859, Pringsheim, 1912).

In order to obtain further information concerning the rate of agar decomposition by the agar-liquefying organism, especially as influenced by the nitrogen source, the results of another experiment will be reported here (table 5). The medium con-

sisted of sea water containing 1 per cent agar, 0.1 per cent K_2HPO_4 and 0.2 per cent of the nitrogen source. Two hundred cubic centimeter portions of the medium were placed in 500 cc. flasks; these were sterilized, inoculated with one strain of the organism, connected with the aeration apparatus and incubated for 90 days.

The results show that the extent of decompositon of agar by the agar-liquefying organism depends to a large extent upon the nitrogen source. In the absence of available nitrogen, very little agar was decomposed, as indicated by the lack of liquefac-

TABLE 5

Influence of nitrogen source upon the amount and rate of decomposition of agar by an agar-liquefying bacterium

NITROGEN SOURCE	TREATMENT	REDUCING SUGAR ON HYDROLYSIS		CO_2 GIVEN OFF		SOLUBLE INORGANIC NITROGEN		pH
		Left	Decomposed	Total amount	Above control	Left	Consumed	
		mgm	*mgm.*	*mgm. C.*	*mgm C*	*mgm.*	*mgm.*	
None..................	Control	1,342	0	21 4		0	0	7.0
	Inoculated	1,291	51	43.3	21.9	0	0	7.0
$(NH_4)_2SO_4$	Control	1,334	0	21 6		79.2		7.0
	Inoculated	990	344	160.1	138.5	72 8	6.4	6.4
$NaNO_3$	Control	1,348	0	20 4	0	58.4		7.0
	Inoculated	602	746	214 0	194.0	34.1	24 3	6 9

tion, only a trace of CO_2 being given off above the control, and by the limited reduction of the hemicellulose content in the agar. This fact proves that the organism is unable to use the combined nitrogen of the agar, as well as that it is unable to fix any atmospheric nitrogen. The ammonium salt was found to be an inferior source of nitrogen for the bacteria as compared with nitrate. Although a considerable amount of agar was decomposed even in the presence of the ammonium salt, the amount of cell substance synthesized was considerably less, as shown both by the limited amount of nitrogen consumed and the larger amount of carbon

liberated as CO_2, when compared with a similar amount of decomposition in a much shorter period of time by culture 1 in the results reported in table 3.

The nitrate proves to be the best source of nitrogen for the organism. More than twice as much carbohydrate is decomposed, as shown by the reduction in hemicellulose content. On comparing these results with those reported in table 3, it is found that in this experiment there was more carbohydrate decomposed for practically the same amount of nitrate-nitrogen assimilated. This is no doubt due to the longer incubation period, which probably leads to the autolysis of some of the cells with the result that some of the nitrogen liberated is again assimilated by the organism.

DISCUSSION

Agar-agar can be considered as a hemicellulose complex, including under the term "hemicellulose" those carbohydrates which are readily hydrolyzed by dilute acids, giving reducing sugars. Recent investigations have brought out the fact that most hemicelluloses so far known are not pure polysaccharides, but consist of mixtures of hexosans, pentosans and uronic acids. Agar-agar is such a hemicellulose, consisting largely of galactan, with an admixture of some pentosan and some uronic acid, as well as certain inorganic salts. It has been shown elsewhere (Waksman and Diehm, 1931) that not all hemicelluloses are decomposed alike by microörganisms. Some, like the mannans and certain pentosans, are very readily decomposed by a large number of microörganisms, while others, like the galactans, are very resistant to decomposition. Some of the hemicelluloses are more resistant to decomposition by microörganisms than the true cellulose. Agar-agar consists largely of a resistant hemicellulose, which is decomposed only by a few specific microörganisms, largely certain bacteria and actinomyces.

A study of the decomposition of agar by a bacterium which occurs in great abundance in sea mud, especially along the coast of various islands in the Bahamas and elsewhere, revealed the fact that the hemicellulose in agar can be readily utilized as a

source of energy. In the process of agar decomposition, the bacterium synthesizes large quantities of microbial cell substance and consumes large quantities of nitrogen. The bacterium can also act upon other polysaccharides, such as starches and mannans, and produce large quantities of sugar from these. This fact is of considerable interest, since it has a bearing upon its possible symbiosis with other organisms, which can use this sugar as a source of energy, such as nitrogen-fixing bacteria, thus supplying both the agar-liquefying organisms and possibly the higher plants with available nitrogen.

The growth of sea-weeds in a medium so poor in available nitrogen, as the sea, thus becomes possible. This enables us to propose the following hypothesis:

The algae growing abundantly synthesize large quantities of hemicelluloses.

↓

The agar-liquefying and other similar bacteria decompose these hemicelluloses liberating large quantities of available energy.

↓

The nitrogen-fixing bacteria use this energy and fix atmospheric nitrogen.

↓

The algae as well as the agar-liquefying bacteria use this nitrogen, directly, or after the bacterial cells have autolized or undergone decomposition.

SUMMARY

A bacterium capable of liquefying agar was isolated from marine sediments around Williams Island. This bacterium was found to occur in large numbers in the sediment.

The agar-liquefying bacterium was found to attack rapidly the hemicellulose complex of the agar and use it as a source of energy. A large part of the carbon was liberated as carbon dioxide and a part was utilized by the organism for the synthesis of bacterial cell substance.

For the decomposition of the agar, the bacterium needs a

source of nitrogen for the synthesis of its cell substance. The nitrogen of the agar is not utilized readily. Nitrate nitrogen is a much better source of nitrogen than ammonium salts.

The bacterium is capable of producing an enzyme which hydrolizes mannan and starch to reducing sugars. Under unfavorable conditions of growth, the organism allows the sugar to accumulate.

A theory is suggested to explain the rôle of the agar liquefying bacterium in the cycle of life in the sea.

The organism was isolated from the Marine mud collected during the Expedition to the West Indies, led by Dr. Field of Princeton University. The authors are indebted to Dr. Field for the facilities offered and for the participation in this Expedition. The authors are also indebted to Mr. Reuszer and Mr. Purvis of this laboratory for assistance in making the chemical analyses.

REFERENCES

AOI, K. 1925. Über eine neue agarzersetzende Bodenbakterienart. Vorläufige Mitteilung. Centrbl. Bakt. II, **63**, 30–32.

AOI, K., AND ORIKURA, J. 1928. On the decomposition of agar, xylan, etc. and the sugars related to these hemicelluloses by *Vibrio andoi* (n. sp.). Centrbl. Bakt. II, **74**, 321–333.

BIERNACKI, W. 1911. *Bacterium Nenckii* Biern., ein neuer den Agar verflüssigender Mikroörganismus. Vorlaufige Mitteilung. Centrbl. Bakt. II, **29**, 166–169.

CZAPEK, F. 1913. Biochemie der Pflanzen, **1**, 643.

DREW, G. H. 1915. On the precipitation of the calcium carbonate in the sea by marine bacteria, and on the action of denitrifying bacteria in tropical and temperate seas. Carnegie Institute, Washington, **5**, 7–45.

FAIRBROTHER, F., AND MARTIN, H. 1923. The swelling of agar-agar. Jour. Chem. Soc., **123**, 1412–1424.

FELLERS, C. R. 1916. The analysis, purification and some chemical properties of agar-agar. Jour. Ind. Engin. Chem., **8**, 1128–1133.

FORBES, E. B., BEAGLE, F. M., AND MENSCHING, J. E. 1913. Mineral and organic analyses of foods. Ohio Agr. Exp. Sta. Bull. 255.

GRAN, H. H. 1902. Studien uber Meeresbakterien. II. Über die Hydrolyse des Agar-Agars durch ein neues Enzym, die Gelase. Bergens Museum Aarbog, No. 2, 1–16.

GRAY, P. H. H., AND CHALMERS, C. H. 1924. On the stimulating action of certain organic compounds on cellulose decomposition by means of a new aerobic microörganism that attacks both cellulose and agar. Ann. Appl. Biol., **11**, 324–338.

HAAS, P. 1921. Carragen (*Chondrus crispus*). II. The occurrence of ethereal sulfates in the plant. Biochem. Jour., **157**, 469–476.

LUNDESTAD, J. 1928. Über einige an der norwegischen Küste isolierte Agar-spaltende Arten von Meerbakterien. Centrbl. Bakt. II, **75**, 321–344.

NEUBERG, C., AND OHLE, H. 1921. Über einen Schwefelgehalt des Agars. Biochem. Ztschr., **125**, 311–313.

PANEK, M. K. 1905. Bakteriologische und chemische Studien über die "Barszcz" genannte Gärung der toten Rüben. Bull. Acad. Sci. Cracovie, **1**, 5–49.

PAYEN, C. R. 1859. (Reference by Fellers.)

PRINGSHEIM, H. 1912. Über den fermentativen Abbau der Zellulose. Ztschr. physiol. Chem., **78**, 266–291.

PRINGSHEIM, H. 1912. Über den fermentativen Abbau der Hemicellulosen. I. Ein Trisaccharid als Zwischenproduct der Hydrolyse eines Mannans. Ztschr. physiol. Chem., **80**, 376–382.

PRINGSHEIM, H., AND E. 1910. Über die Verwendung von Agar-Agar als Energiequelle zur Assimilation des Luftstickstoffs. Centrbl. Bakt. II, **26**, 227–231.

SAMEC, M., AND SSAJEVIC, V. 1922. Koll. Chem. Beihefte, **16**, 285–300.

VAN DER LEK, G. B. 1929. *Vibrio agarliquefacians* Gray. Ned. Tijdschr. Hyg., Microbiol., Serol., **3**, 276–280. (Ref. Centrbl. Bakt. II, **79**, 435–436.)

WAKSMAN, S. A., AND DIEHM, R. A. 1931. On the decomposition of hemicelluloses by microörganisms. Soil Sci., (to be published).

CULTIVATION OF RICKETTSIA-LIKE MICROÖRGANISMS FROM CERTAIN BLOOD-SUCKING PUPIPARA

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INTRODUCTION

Rickettsia or rickettsia-like organisms have been studied extensively during the last decade; but there is as yet no clarity as to the nature and precise definition of this group of microbes. At least five pathogenic and a large number of non-pathogenic species have been described in a large variety of blood-sucking as well as non-blood-sucking insects (Hertig and Wolbach (1924), Cowdry (1923), Weigel (1924)). These descriptions are, however, based entirely or chiefly on morphologic studies, and it is more than likely that at least part of the pleomorphism ascribed to rickettsia is due to a confusion of a variety of organisms, including so-called rickettsias as well as bacteria belonging to the group of insect symbionts.

Da Rocha Lima (1916) was the first to describe a peculiar organism which he found in the gut of lice fed on typhus patients. These organisms were elliptical, smaller than *B. melitensis*, and were stained reddish with Giemsa. They were named *Rickettsia Prowazeki*. The primary characteristic ascribed by Da Rocha Lima to these organisms was their power to penetrate the cells of the wall of the insect intestines and multiply, and consequently he maintained that only organisms having this property, in addition to the peculiar morphology and staining reaction, can be classed in this group.

The large amount of work published subsequently and summarized by Hertig and Wolbach (1924) and Cowdry (1923) made it abundantly clear that rickettsia-like organisms were found in a

large number of arthropods.   Hertig and Wolbach, after a comprehensive critical review of the literature, suggest that the term Rickettsia be limited to intracellular pathogenic organisms in the sense originally defined by Da Rocha Lima.   But this definition is too limited, since in almost every group of microörganisms there are pathogenic and non-pathogenic species in the same genus.   These authors admit that such a limitation is at the moment difficult to make and state that "in the meantime 'rickettsia' will doubtless continue to be a loose but convenient group-name for certain minute microörganisms associated with arthropods."

The other characteristics are even more difficult to circumscribe.   The minuteness of size, characteristic staining reaction with Giemsa and association with insect hosts are fairly constant characteristics.   But wide divergence can be noted in the description of rickettsias found in the literature.   One of the most confusing elements is the extreme pleomorphism ascribed to these organisms.   The organism described by Arkwright, Atkin and Bacot (1921) in the bed bug as *R. leticularia* is extremely pleomorphic, and this pleomorphism has since been considered by them, as well as by other investigators, as a peculiar characteristic of the group.   This pleomorphism also forms the basis of the proteus theory of the etiology of typhus which has been developed particularly by the researches of Kuczinsky (1922).   Anigstein (1927) in his attempt to repeat the work of Noeller with *R. melophagi* also describes a variety of forms under the name Rickettsia, ranging from minute coccoid-like bodies to filamentous mould-like forms.   He maintains with Kuczinsky that the rickettsia forms are only a stage in the complex life cycle of these organisms.

The extensive studies by Buchner (1930) and his pupils which showed that many insects harbour bacterial symbionts and that the same species may be regularly inhabited by more than one organism furnish an explanation of the confusion existing in the literature concerning the pleomorphic character of Rickettsia. It may well be that such symbionts, which in most cases differ widely in their morphology from rickettsia, have been responsible for the extreme pleomorphism ascribed to the latter.   Thus, the

organism described by Arkwright and his associates in the bed bug is most probably a symbiont and not a rickettsia. It has also been shown (Zacharias (1928), Aschner (1931)) that the pleomorphic organisms of the sheep ked pictured by Arkwright and Bacot (1921) and by Anigstein (1927) consist of two entirely distinct forms of bacteria which regularly inhabit this insect. Hertig and Wolbach (1924) were the first to attempt to differentiate between rickettsia and symbionts. They define rickettsias as Gram-negative, intracellular, minute, coccoid or diplococcoid organisms, $0.3-0.5\mu$; staining readily with Giemsa but poorly with other aniline stains, without any well defined contour and difficult to cultivate *in vitro*. These authors evidently do not consider extreme pleomorphism as a dominant characteristic of rickettsia. This definition is today the best available, except that it is doubtful whether we are justified in limiting the group to intracellular types only, since, as was pointed out above, a variety of accepted extracellular forms have been described (*R. quintana, R. melophagi*) which have the characteristics of the group. Cowdry also arrives at the conclusion that at present the only criterion for distinguishing the rickettsias from other insect organisms is their minute size.

It is apparent from a review of the literature that there is a large group of microörganisms specifically adapted to insect hosts. Many of these have a definite relation to the biology of their host, as shown by the mutual adaptation of the microbes and the insect. Such forms are often highly pleomorphic, intracellular, and regularly transmitted from mother to offspring. These constitute the so-called symbionts. Of these symbionts no forms pathogenic to vertebrates are as yet known. There is another, more circumscribed and well defined group of organisms which is less definitely adapted to the insect host, and presumably plays no part in the normal biology of the insect. These correspond to the description usually given of rickettsia. Of this group some members are pathogenic for their host (*R. Rocha-Lima, R. prowazeki*), and others are pathogenic for mammals. A proper definition or differentiation of this latter group cannot be reached on the basis of morphologic characteristics alone. Progress in the study of

these organisms can be obtained only by a systematic study and cultivation of the microbic flora of the parasitic insects. Such studies would on the one hand extend our knowledge of the biologic properties of the group as a whole, and on the other define more specifically the characteristics of the genus Rickettsia. The systematic study by Noguchi (1923) of the microbic flora of the tick, *Dermacentor andersoni*, is illuminating in this connection. Noguchi was able to cultivate three distinct species of bacteria, none of which, however, proved to be the virus of spotted fever.

With this in view we have undertaken a study of the symbionts and rickettsias of a number of species belonging to the pupipara. The morphologic studies relating to symbionts and their biologic significance will be published elsewhere (Aschner (1931)). Attempts to cultivate the symbionts have not met with success. However, we have succeeded in cultivating from several different species of pupipara organisms resembling morphologically and tinctorially the rickettsias present in these insects. The purpose of this paper is to report the results of these experiments.

MATERIAL AND METHODS

Eight species of pupipara were studied, but the cultivation experiments were confined to four. These were *Melophagus ovinus*, the common sheep ked, *Lipoptena caprina*, a parasite of the goat, *Hippobosca equina* and *Hippobosca capensis* parasites of the horse and dog respectively.

The pupipara are blood sucking diptera, characterized by the fact that the larvae develop in the insects and pupate a few hours after they are laid. They are extremely specific parasites and are adapted only to a given host; seldom does it occur that an insect can be transferred from one host to another. Well-defined rickettsia-like organisms have been found in seven of the eight species studied. These organisms are not present, always, in every insect. In the morphological studies, both intracellular and extracellular rickettsias have been noted. Intracellular forms were found in *Nycteribosca kollari*, *Nycteribosca biarticulata* and *blasii* (bat) and *Lynchia maura* (pigeon); they occurred chiefly in the epithelial cells of the gut and in the cells of the mal-

pighian tubules, arranged singly or in small clumps. The extra-cellular forms were found in the lumen of the gut, usually massed on the epithelial cells. These forms were not equally abundant in all insects; they were most abundant in *Lipoptena caprina* and *Melophagus ovinus*, less so in *Hippobosca capensis* and only rarely in *Hippobosca equina*. In addition to the rickettsia-like organisms there were also noted large pleomorphic symbionts, and in some cases also small Gram-negative bacteria.

The rickettsia-like organisms noted in these insects resemble the symbionts in that they are transmitted to the larvae. Noeller has established this fact in the case of *Melophagus*, and one of us (Aschner (1931)) was able to demonstrate that this was also the case in five other species of those studied by us. As was first shown by Zacharias (1928) the extracellular rickettsias of *M. ovinus* do not invade the eggs but the larvae become infected from the milk glands. This seems to be true also for the other species. The number of rickettsias in the young adults is usually small, and they become particularly numerous after a blood feed. The intracellular rickettsias infest the eggs and are transmitted in that way.

Noeller was the first to report the successful cultivation of the rickettsia-like organisms from the sheep ked, *Melophagus ovinus*. He described on his blood agar plates gray, transparent colonies 0.4 to 0.6 mm. in diameter. The organisms were uniform in ap-pearance and resembled those seen in the insect host. Jungmann (1918) and Hertig and Wolbach (1924) were subsequently able to confirm these findings. The important fact is that the culture forms described by these authors did not show wide morphological variations, and, only in older cultures, did Jungmann observe larger, more intensely stained, spherical or pear-shaped forms. These types have also been observed by us in our cultures, and probably represent degeneration forms. Anigstein (1927) who also attempted to confirm Noeller's work reports results which differ in many essentials from those of the other workers. He received at first dew drop colonies resembling those described by Noeller and by Hertig and Wolbach; later, however, these colonies lost their transparency and developed a greenish or yellow tint.

In contrast with the sparse growth obtained by the other authors he succeeded in getting an abundant growth. Furthermore, his cultures were pleomorphic, and he described a variety of strains which differed morphologically, culturally and serologically from one another. The relation of these findings to our own results will be discussed below.

The methods employed in this investigation were briefly as follows:

Morphological examination of insect. For sections, the insects were fixed in Regaud or Carnoy fixative. For demonstration of rickettsia-like organisms the former fixative is the most satisfactory. The sections were stained with Giemsa by the Noguchi method. Smears of guts or feces were fixed in 95 per cent alcohol, stained for thirty minutes with Giemsa and washed with distilled water. The organisms take the stain readily. When there were large masses of organisms, the ordinary Gram stain could be used.

Methods of cultivation. For the cultivation experiments the first problem was to free the insects of external bacteria. The following procedure proved entirely satisfactory. The insects were dipped in a 5 per cent tincture of iodine for five to ten seconds, then washed in 95 per cent alcohol for the same length of time, and then rinsed in sterile saline. The insects were then placed on sterile slides under the dissecting microscope, the abdomen cut off and the contents of the intestines pressed out into a drop of sterile saline solution. The abdomen was then transferred to a second sterile slide, the intestines teased out and separated from the sex organs. All, or parts, of the intestines were then placed on Noeller blood agar plates or into the fluid medium. The cultures were incubated at 26°C. and observed for a period of at least two weeks.

A check of the satisfactory character of the sterilizing procedure was furnished by the peristaltic movements of the gut as well as by the crithidia of the goat trypanosome present in the goat parasite. These crithidia grew readily in some of the media tested, and consequently served as an index of the toxic effect of the sterilizing procedure. It appeared that the iodine-alcohol

treatment sterilized the exterior of the insects without in any way affecting the viability of the organisms present in the gut.

It was our object in these experiments to grow only those organisms found in the insects which appeared tinctorially and morphologically to correspond to the rickettsia-like forms. We did not attempt, therefore, to classify other types of bacteria which from time to time grew out on one or another of the media used.

Media employed. A large variety of media were tested. Among these were Noeller's blood agar plates and Noguchi's media, as well as many others. We shall here confine ourselves to a description of those media which proved most useful in the cultivation of those rickettsia-like organisms which failed to grow on any other culture media. On these media closely related organisms were cultivated from all of the insects studied, but repeated effort failed to bring about their adaptation to the more common culture media.

Peptone-gelatine-blood medium. This medium consisted of a solution of salts, peptone and gelatine. A stock solution was prepared consisting of 10 grams peptone, 10 grams gelatine and 100 cc. water. One cubic centimeter of this solution was diluted in 10 cc. Locke solution, saline or glucosal. At first these mixtures were adjusted to pH 7.3, and sterilized in the autoclave; later it was found that a reaction of pH 6.6 was more favourable. After autoclaving, the media were divided into specially cleaned neutral test tubes, 2 cc. per tube. A day or two before use 0.25 cc. of defibrinated sheep or goat blood was added to each tube.

This medium proved more satisfactory than blood broth, particularly for primary cultures. On the one hand it is not favourable for the growth of trypanosomes which overgrow the rickettsia in blood-broth cultures; on the other the red cells lake less readily than in the broth.

Locke-semisolid. This medium consists of a mixture of 1 part nutrient agar, 2 parts defibrinated rabbit blood and 6 parts Locke solution. This medium is suitable for maintenance of strains, but is not satisfactory for primary cultures.

Noeller-blood agar. This medium consists of two parts agar

and one part blood. The blood is added while the agar is at 80°C. giving the medium a chocolate-brown appearance. Noeller first cultivated the *R. melophagis* of the sheep ked on this medium. We were able to obtain growth on plates around the bits of insect intestine. Sub-cultures on the solid medium were, however, difficult and only rarely successful, except in the case of the horse strains.

CULTIVATION EXPERIMENTS

As stated above, cultivation experiments were made with four species of insects. From all these species an organism was culti- vated which had characteristic cultural and morphological prop- erties and which resembled morphologically and tinctorially those seen in the guts of the insects.

Morphology and staining. All the organisms isolated were minute bacilli, often coccoidal in shape, discrete or in large masses. Although of somewhat variable morphology the range of size was not greater than 0.5 to 0.3. They were all Gram-nega- tive and stained uniformly with Giemsa, taking a violet tint. No bipolar staining was noted. In older cultures the staining was irregular, the tints being violet, pink and blue; the same clump contained also larger pear-shaped or coccus-like intensely stained organisms. Young cultures were, however, morphologically and tinctorially homogenous.

Biological characters. Successful results were obtained only in liquid media, the most uniform results being obtained in the pep- tone-gelatine-blood medium. The cultures were incubated at 26 to 28°C. The growth was localized on the surface of the red cells at the bottom of the tube, while the supernatent fluid re- mained entirely clear and unchanged. When growth was abun- dant there could be noted a greyish-white net-like deposit or else small discrete whitish pin point colonies on top of the red cells. This whitish film resembled a layer of white cells, and could easily be mistaken for it. Red blood cells were essential for growth. Hemoglobin as such was not sufficient, and if the cells hemolized too quickly no growth occurred. Probably for this reason sheep or goat cells which we found less subject to lysis than rabbit cells

proved more satisfactory. The amount of blood did not appear to be of great importance, provided that there was enough to form a layer of cells at the bottom of the tube which could serve as a matrix for the growth of the organisms.

Growth was at the best very slow. Usually, seven to ten days were required for growth to appear, and good growth could be noted only after two weeks.

On solid media growth was scarcely visible, and often the colonies were so minute that growth could only be demonstrated by staining. None of the strains, except the ones from *Hippobosca equina*, could be maintained on solid or semi-solid media for more than two generations. The horse strain was the only one that grew on glucose-blood-agar slants, but the growth developed slowly and never became abundant, even after many sub-cultures.

Transfers had to be made fairly frequently. The best interval for sub-culture was two weeks. Often, however, sub-cultures failed without any reason at all, although every care was taken to keep the composition of the media constant and uniform.

Efforts to determine the fermentative characters of these organisms were not successful. At best the growth was relatively so scanty as to produce no measurable change in the pH of the media. Glucose and glycerol appeared to have a favourable effect on the growth, but no fermentative action could be detected.

None of the organisms had any pathogenic effect on test animals. Large amounts of culture were inoculated into mice and guinea pigs without any noticeable effect, either on the temperature or blood picture. Attempts to recover the organisms from the peritoneal fluid or blood of the inoculated animals at various intervals after the inoculation yielded negative results.

The relation of these organisms to those cultivated by other investigators, notably Noeller (1923), Jungmann (1918), Hertig and Wolbach (1924), and Anigstein (1927) cannot be stated with certainty. Morphologically and tinctorially they resemble Rickettsia and correspond with Noeller's description of *R. melophagi*. Unlike Noeller's strain the organisms cultivated by use from the sheep ked failed to grow on solid media. They differed, however, both culturally and serologically from two of the strains culti-

vated by Anigstein, which he was kind enough to send us. These cultures grew readily on blood agar, giving a heavy yellowish growth; they were also Gram-negative, but on the whole larger than those grown by us. Whether this difference in size is a real one or due to the difference in media it is difficult to say; but the gross cultural differences were striking. Serologically, too, they proved to be entirely unrelated. Immune sera produced by our strain isolated from *Melophagus ovinus* had no effect on Anigstein's cultures isolated from the same species of insect.

The relation of the organisms cultivated by us to those found in the insect must also for the present remain a matter of conjecture. The morphologic and tinctorial resemblances were striking, as can be seen from plates 1 and 2. Serologic comparisons did not yield any clear cut results. Moreover, in the case of *Lipoptena* it was possible to recover the same organism in 30 out of 40 insects cultured at different times and on the different media described. This is a reasonable indication that we were dealing with the same organism as that present in the insect.

But whether the organisms cultivated were identical with, or only one of, those noted in the insect it is clear that we have cultivated from a variety of pupipara insects organisms which appear closely related to one another culturally, morphologically and tinctorially, and bear a close resemblance to the rickettsias found in the insects. It is our purpose to extend these methods to the study of the microbic flora of insect vectors of pathogenic rickettsias.

DISCUSSION

Our studies have now extended over a period of two years. During this time many insects have been examined and a large number of cultures made. The work is extremely difficult, but our observations thus far have convinced us that further advances in our knowledge of this group of microbes depends on a greater familiarity with the flora of insect parasites in general, and, more particularly, with the group of organisms having the general characteristics of Rickettsia. Knowledge of the cultural properties of the non-pathogenic members of this group of microbes may aid in the study of the pathogenic members of the group.

It is essential at the outset to differentiate between the symbionts and rickettsia-like organisms, both of which are adapted to the insect host; and between these and chance invaders. Furthermore, it should be emphasized that the symbionts differ essentially from Rickettsia. The former represent a wide variety of forms some of which definitely belong to the bacteria, and others to the higher bacteria and moulds and like them show extreme pleomorphism and irregular staining reactions. The bacillus cultivated by Glaser (1930a and b) from the American and German cockroach, for example, belongs to this group. It is a Gram-positive pleomorphic diphtheroid resembling morphologically and tinctorially the intracellular organisms seen in the insect. The mere fact that they are found in the insect cells is in our opinion no justification for classing them with the entirely distinctive group of Rickettsia.

The rickettsias have a much more uniform morphology and size, and are all Gram-negative. The work of Anigstein stands unconfirmed, and our own observations have convinced us that this investigator obtained a variety of organisms which he unjustifiably considered different aspects of the same species. Unless future studies prove the contrary, the present indication is that the more conservative view that the group is fairly stable within narrow limits appears to be more in accord with the facts. Ascribing extreme pleomorphism to an organism on the basis of appearances in insects known to harbour other organisms leads to confusion and not to a precise delimitation of the group.

SUMMARY

Experiments are reported dealing with the cultivation of extracellular non-pathogenic rickettsias from a number of pupipara. Methods are described by means of which cultures of Rickettsia were obtained repeatedly from the parasitic pupipara of the sheep, goat, horse and dog. The organisms obtained in culture were minute, Gram-negative, coccoidal rods corresponding to the usual description of Rickettsia and resembling the forms seen in the guts of the insects.

REFERENCES

ANIGSTEIN, L. 1927   Arch. f. Protistenkunde, **57**, H.2.

ARKWRIGHT, A., ATKIN AND BACOT, A. 1921   Parasitol., **13**, 27.

ARKWRIGHT, A. AND BACOT, A. 1921   Transact. of the Royal Soc. Trop. Med., **15**, 14.

ASCHNER, M. 1931   Zeitschr. f. -orphol. und Oekol. d. Tiere, **20**, 368.

BUCHNER, P. M. 1930   Tier und Pflanze in intrazellularer Symbiose, (second edition), Berlin, 1930.

COWDRY, E. V. 1923   Jour. Exper. Med., **37**, 431.

DA ROCHA LIMA, H. 1916a   Arch. f. Schiffs- u. Trop. Hyg., **21**, 17; 1916b, Berliner Klin. Wochenschr., **53**, 567.

GLASER, R. W. 1930a   Jour. Exper. Med., **51**, 59.

GLASER, R. W. 1930b   Ibid., **51**, 903.

HERTIG, M. AND WOLBACH, S. B. 1924   Jour. Med. Res. **44**, 329.

JUNGMANN, P. 1918   Dtsch. Med. Wochenschr., **44**, 1346.

KUCZYNSKI, M. 1922   Klin. Wochenschr., **1**, 1412.

NOELLER, W., UND KUCHLING, M. 1923   Berlin. Tieraerztl. Wochenschr., **30**, 197.

NOGUCHI, H. 1923   Jour. Exper Med , **37**, 221.

WEIGEL, R. 1924   Klin. Wochenschr., **3**, 1590.

ZACHARIAS, A. 1928   Zeitschr. f. Morphol. und Oekol. d. Tiere, **10**, 676.

PLATE 1

FIG. 1. Section through the intestine of *Hippobosca capensis* Olfers, showing intracellular symbionts at the bases of epithelial cells as well as a few rickettsia on the surface of the middle cell.   Fixation, Regaud; stain, Giemsa; ca. 700×.

FIG. 2. Section through the intestine of *Lipoptena caprina* Austen showing a thick layer of rickettsia covering the epithelial cells.   Fixation, Regaud; stain, Giemsa; ca. 700×.

FIG. 3. Smear of faeces of *Hippobosca capensis* Olfers with numerous rickettsia. The filamentous organism is a symbiont.   Stain, Giemsa; ca. 700×.
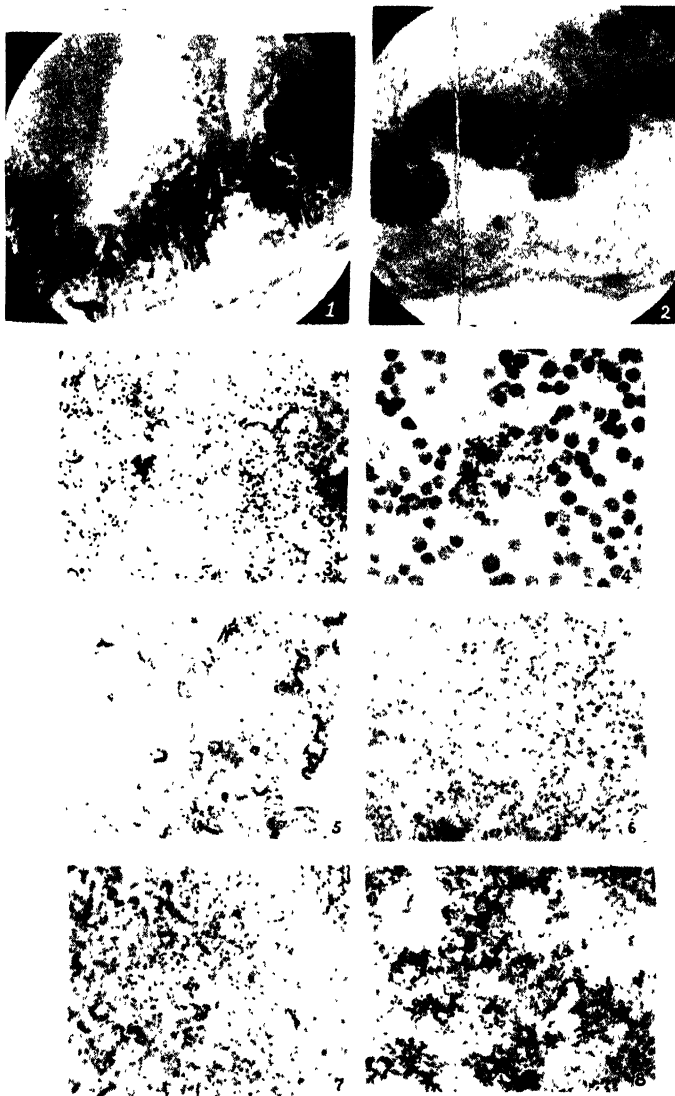
FIG. 4. Rickettsia from *Hippobosca capensis* Olfers in a Locke-peptone-gelatine-sheep-blood culture; note irregular staining of the clump.   Stain, Giemsa; ca. 700 ×.

FIG. 5. Rickettsia and symbionts of *Lipoptena caprina* Austen in a feces preparation.   Stain, Giemsa; ca. 600×.

FIG. 6. Rickettsia from *Lipoptena caprina* Austen from a blood agar culture. Stain, Giemsa; ca. 700×.

FIG. 7. *Rickettsia melophagi* from the intestine of *Melophagus ovinus*.   Stain, Giemsa; ca. 700×.

FIG. 8. *Rickettsia melophagi* in a Locke-peptone-gelatine-sheep-blood culture. Stain, Giemsa; ca. 700×.

PLATE 2

FIG 1   Rickettsia from *Hippobosca equina* L. in a Locke-peptone-gelatine culture, showing the formation of large intensely stained bodies described by Jungmann in *R Melophagi* cultures   Stain, Giemsa; ca 1000×

FIG 2   The same in a blood culture of *Rickettsia melophagi*   Stain, Giemsa; ca. 1000×.

FIG. 3   *Hippobosca capensis* strain in a sheep-blood-bouillon culture   Uniform growth   Stain, Giemsa; ca 1200×

FIG 4   *Lipoptena caprina* strain in a goat-blood-bouillon culture   Streptococcus-like chain-forming growth.   Stain Giemsa; ca 1200×.

FIG 5.   *Hippobosca equina* strain on glucose-blood-agar.   Stain, Giemsa; ca. 1200×.

FIG 6   *Melophagus* strain in a sheep-blood-bouillon culture   Stain, Giemsa; ca 1200×.

FIG. 7   Aningstein s strain C. 17 from *Melophagus ovinus* on blood agar; contrast with the various strains of rickettsia   Stain, Giemsa; ca 1200×.

(I. J. Kligler and M. Aschner   Rickettsia-like microorganisms.)

THE RECOVERY OF BACTERIOPHAGE FROM FILTRATES DERIVED FROM HEATED SPORE-SUSPENSIONS

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In recent papers by den Dooren de Jong (1930, 1931) dealing with bacteriophages for *B. megatherium* the author states that of eighteen megatherium strains which he was studying three were lysogenic, that is, their broth culture filtrates contained bacteriophages capable of causing lysis of other susceptible strains. Spores of the parent strains could survive exposure to a temperature of 100°C. for five minutes, and the filtrates of the cultures derived from these heated spores showed all of the typical bacteriophage properties. The lytic principle itself was killed or inactivated by heating at 70°C. for five minutes. On the basis of these experiments he concluded that, as the bacteriophage alone was destroyed at 70°C., the principles found in the cultures arising from the heated spores must have been developed by the living cell itself and that, therefore, d'Herelle's theory of the living nature of the bacteriophage is not tenable.

This conclusion seems warranted if we admit that all bacteriophage present in a culture is actually destroyed by heating at 90 or 100°C. The principle, when free, undoubtedly is so destroyed or inactivated, but if some of it is incorporated in the spores when they are formed, it is not impossible that it may be protected by the same mechanism, whatever it is, that enables the spore to withstand temperatures lethal to vegetative cells. This would permit an alternative explanation.

If the latter assumption is true, the lysogenic strains mentioned above may have been contaminated with bacteriophage when

they were originally received. Such strains are not common, for only three were found among the 18 studied, and an equal number of cultures obtained from various sources and examined in this laboratory have all been bacteriophage-free when tested against two susceptible strains, No. 1 and No. 338b.

Through the courtesy of Dr. den Dooren de Jong there were secured several of his megatherium strains, including one, No. 899, which apparently produced bacteriophage, and the sensitive No. 338b, and his experimental results were readily verified. It seemed advisable, therefore, to determine whether or not lysogenic strains in which the lytic agent was carried through the spores could be produced from known bacteriophage-free strains of spore-forming organisms.

For this purpose the following organisms and their homologous bacteriophages were studied. *B. megatherium* and *B. anthracis* (Cowles, 1930; 1931), a strain of *B. subtilis* obtained from Dr. d'Herelle, and *Cl. tertium*. Incidentally, the bacteriophage for *Cl. tertium* is the first to the writer's knowledge that has been found for any of the clostridia. Its isolation is of some interest since it confirms what has always been assumed—that the phenom-enon of transmissible autolysis is present among members of this group as well as among the aerobes.

With *B. megatherium* and *Cl. tertium*, results were obtained indicating that after proper contacts between organism and bacteriophage, the latter could be carried through the heated spores, but for several reasons these two species were not so well suited to demonstration purposes as *B. anthracis* and *B. subtilis*, to which, accordingly, attention was more particularly directed.

The Strasbourg strain of *B. anthracis* was used. This is easily lysed, but develops within twenty-four hours a secondary growth which is resistant to the lytic action. Resistance is maintained through numerous cycles of spore formation, even when the spores are heated between each cycle to kill vegetative cells and free bacteriophage, and furthermore bacteriophage can usually be demonstrated in the filtrates of such cultures. In several instances spore suspensions were sealed in glass ampoules and subjected, in the water-bath, to temperatures of 80, 85, 90, 95°C. for

ten minutes and to 100°C. for five minutes.   Following the heating, small amounts from each tube were plated and the remainder inoculated into broth.   In every case abundant growth resulted from the samples heated at 80, 85, and 90°C., but only a few spores, if any, survived 95°, and none 100°C.   Filtrates from broth cultures of the first three consistently showed lytic power. Those from cultures of the 95° specimens did not.   Forty colonies were picked from the various plates and inoculated into broth, but in no case could bacteriophage be demonstrated in the filtrates, although all 40 sub-strains were resistant to lytic action.   Mass inoculations from the plates, however, did show the presence of the lytic agent.   Apparently the percentage of spores of this strain, so modified that they carried or produced bacteriophage, was relatively small at this stage in its history.

With the strain of *B. megatherium* obtained from Dr. de Dooren de Jong (No. 899) this was not the case.   Spores of this strain, subjected to the same treatment showed ready survival at 80, 85, and 90°C., but not at 95° or 100°C.   All broth culture filtrates of the first three, and all colonies picked from plates seeded with the heated spores showed the presence of bacteriophage.   This particular strain seems to have been well stabilized in its relationship to the lytic agent.

The subtilis strain differed somewhat from the above in its characteristics.   The race of bacteriophage used was of great potency and caused complete and permanent lysis.   A resistant form of growth, however, was obtained by heavily seeding an agar plate with organisms, streaking it with bacteriophage, and then culturing on agar the growth which resulted along the bare lysed area.   Several transfers of this resistant variant gave a strain, the spores of which seemed to carry bacteriophage just as had those of *B. anthracis* and *B. megatherium*.   The subtilis spores could withstand a temperature of 90°C. for ten minutes or 100°C. for five minutes, and the growth resulting from these spores contained bacteriophage.   Some, but not all, of the colonies picked from plates seeded with such heated spores gave cultures yielding lytic filtrates.

Some evidence was obtained, particularly with the anthrax strain, that repeated sporulation, heating and growth, weakened or eliminated the bacteriophage (or the ability to produce it), without affecting the resistance of the cells to the lytic agent.

Tests on the thermal death points of the bacteriophages used in these experiments showed that 75°C. for ten minutes was sufficient to inactivate them, indeed, the lytic power of the anthrax bacteriophage was destroyed at 60°C. in that time.

The observations reported above indicated that den Dooren de Jong's results in his experiments may bear another interpretation.

Since heating spores to temperatures of 80, 90, or 100°C is not necessarily sufficient to render them bacteriophage-free a fact which has been tacitly assumed or explicitly stated in most papers dealing with this subject, it follows that lysogenic strains may very well have been developed by contact with bacteriophage at some stage in their history.   Experiments designed to show that bacteriophage can arise spontaneously in a culture have always been criticized rather severely, although some of the evidence is very suggestive and has been summarized by Hadley. More recently Smith and Jordan have reported observations on a culture of *C. diphtheriae* in which, at irregular intervals, bacteriophage could be demonstrated, although in the interims none could be found. The difficulties of carrying on such experiments in laboratories where much bacteriophage work is being done are obvious, since the possibilities of contamination, due to the ease with which bacteriophage corpuscles are disseminated, are ever present.

In the experiments reported in this paper it may be, of course, that the bacteriophage as such does not survive the heating process, but that it has so stimulated the bacterial cell that upon subsequent growth the lytic agent is again produced. However, until more is known of the mechanism by which spores are rendered thermotolerant, it does not seem possible to reject without consideration the idea that the bacteriophage, if incorporated in the cell when sporulation occurs, may be protected from the effects of heat by that same mechanism.

SUMMARY

That bacteriophage may be demonstrated in growth resulting from spores which have been heated to a much higher temperature than that tolerated by free bacteriophage was shown by den Dooren de Jong, who on this basis concluded that the lytic principle was generated in the cell.

In this paper evidence is presented to show that known bacteriophage-free strains of several spore-forming organisms may be so changed by the action of the bacteriophage that the principle can be demonstrated in filtrates of cultures developed from heated spores.   This does not necessarily mean that the lytic agent as such survives the heating process, but it does mean that the recovery of a lytic principle from a pasteurized culture is not conclusive proof of the spontaneous generation of bacteriophage.

REFERENCES

COWLES, P. B.   (1930)   The bacteriophagy of a spore-forming organism.   Jour. Bacterial **20**, 15–23.

COWLES, P. B.   (1931)   A bacteriophage for *B. anthracis*.   Jour. Bacteriol, **21**, 161.

DEN DOOREN DE JONG, L. E. (1930)   The bacteriophage of *B. megatherium*.   Proc. Kon. Akad. Wetensch te Amsterdam, **33**, 1–5.

DEN DOOREN DE JONG, L. E.   (1931)   Studien über Bakteriophagie I, II.   Cent. f. Bakt. I, Orig., **120**, 1–23.

HADLEY, PHILIP (1928)   The Twort-d'Herelle phenomenon.   Jour. Infect. Dis., **42**, 263.

HADLEY, P., AND JIMENEZ, B.   (1931)   Production of bacteriophage by enforced dissociation.   Jour. Infect. Dis., **48**, 176.

SMITH, GEO. H., AND JORDAN, E. F.   (1931)   Distribution of diphtheria bacteriophage.   Yale Jour. Biol. and Med., **3**, 423.

COLI-AEROGENES DIFFERENTIATION IN WATER ANALYSIS

II. THE BIOCHEMICAL DIFFERENTIAL TESTS AND THEIR INTERPRETATION

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We have shown in an earlier section of this paper that a larger percentage of routine plates may contain no typical *Bact. coli* or *Bact. aerogenes* colonies, but that these cultures are often mixed and therefore cannot be differentiated without purification. Also that a surprising portion of single isolated colonies, ordinarily considered pure and usually differentiated macroscopically, are not pure. These must be purified before they can be correctly differentiated. We have shown that neither the macroscopic examination of a single streaked plate nor the microscopic examination of a Gram stain smear gives sufficient evidence of the purity of a culture. We have relied largely on certain biochemical differential reactions used in conjunction with the aforementioned tests. The efficacy of the various methods used for purification will be determined to a large extent by the use of biochemical differential methods. We are therefore going to reverse the natural order of sequence and describe the biochemical tests used before we discuss purification.

CHARACTERISTICS FOR DIFFERENTIATION

In order to differentiate the organisms of the coli-aerogenes group it is necessary to use characteristics which are different for the two types and are also stable and not easily variable in the individual species. Bergey (1925) separates the coli section or genus *Escherichia* from the aerogenes section or genus *Aerobacter* on the basis of the acetyl-methyl-carbinol or Voges-Proskauer

reaction.   Kluyver and Donker (1926) use the same reaction as a basis of separation for the group.   However, Buchanan and Fulmer (1930) citing the above investigators say:

> Kluyver and Donker[1] (1926) have also shown that in a suitably buffered medium *Bacterium coli* does not transform all acetaldehyde to ethyl alcohol and acetic acid, but in part to acetyl-methyl-carbinol.   In other words, a modification of the pH of a medium changes the metabolism to that characteristic of *Bacterium aerogenes*.

If the above is true, this basis of classification and separation becomes rather weak.

In his study of microbic dissociation Hadley (1927) reviews much of the work on dissociation of the coli-aerogenes group and shows that it has been repeatedly observed in members of this group.   Unfortunately most of the investigators studied dissociation with respect to cultural, morphological, serological or fermentative characteristics.   There is a lack of data on the other biochemical variations accompanying dissociation in the coli-aerogenes group.   If a simple adjustment in the pH of the medium, as stated by Kluyver and Donker, is sufficient to enable *Bact. coli* to assume a biochemical characteristic of *Bact. aerogenes* we would certainly expect biochemical variations in dissociants of the group.   Hadley has shown that there is a variation in the production of pyocyanin and in the proteolytic power of the dissociate and the mother type of *B. pyocyaneus*.   On the other hand Soule (1928) reports that both the S and R forms of *B. subtilis* produce indol and reduce nitrates.   The fact that Neisser (1906) and also Massini (1907) obtained a "coliform" lactose fermenting organism as a dissociant from a parent culture that could not ferment lactose is rather surprising.   We have isolated a number of coliform cultures which apparently lost their power to ferment lactose after transferring to lactose and replating on an isolation medium several times.   We explain this phenomenon on the basis of symbiotic gas production as shown by Sears and Putnam (1923) and Ishikawa (1927), and the final ascendency and isola-

[1] See note, page 135, for recent opinion of Kluyver and Donker.

tion of one non-lactose-fermenter of a pair in an initially mixed culture, rather than by dissociation. Dulany (1928) reported that *Bact. coli* dissociants had characteristic differences in colony form and cell morphology. Also, though a distinct difference in the virulence of the dissociants was noted, only slight differences in the cultural reactions were found. Dawson (1919) showed that by changing the character of the medium the chemical constitution of the bacteria changed. The chemical composition of the organisms showed considerable variation after they had been grown for about 200 generations on 8 different media. These changes were accompanied by variations in the serological and fermentative reactions so that in some cases the variations amounted to the production of a new strain. Hadley (1927) cites Bergstrand, Hauduroy, Fejgen, Ogata, Grumbach, and Dimtzer as having reported similar fermentative changes in dissociants. From the literature it is difficult to determine whether the more common biochemical reactions of the coli-aerogenes group are any more stable than the colonial, morphological, serological or fermentative characteristics.

Our experience has been that some of these biochemical tests are very stable, cannot be easily changed and are the most reliable characteristics for differentiation of the members of the Coli-aerogenes group. We shall therefore study the following problems in this paper:

1. The various biochemical reactions available.

2. The technique and particular value of the four tests chosen.

3. The stability of this entire group of tests on pure *Bact. coli* and *Bact. aerogenes* strains.

4. The interpretation of the various combinations of these four tests observed on routine cultures.

5. The various methods of purification tried and their value as judged by these biochemical tests for purity.

THE BIOCHEMICAL DIFFERENTIAL REACTIONS

Standard Methods (1925) recommends the methyl-red and Voges-Proskauer reactions, Koser's citrate test and the uric acid test for differentiation of the coli-aerogenes group. Many other

tests have been suggested and have value under special conditions. Some of these are the indol test, proteolytic tests, the chinic acid test (Butcher (1926)) and other organic acid tests, the cellibiose test (Jones and Wise (1926) and Koser (1926)) and the various other carbohydrate fermentation tests. The proteolytic tests and the chinic acid medium are valuable largely in the differentiation of the *Bact. cloacae* and other members of the aerogenes section. This is not so important in water laboratories where it is necessary to limit the number of tests and therefore neither of these tests were included in our routine work. While the reports on the cellibiose test have been favorable, it is rather expensive and therefore impractical for routine laboratory use. The various carbohydrate fermentation tests have long been used in systems of classification of the coli-aerogenes group. Houston (1911) obtained 26 real or apparent varieties of lactose-positive and indol-producing organisms from water when classified by such sugar fermentation tests. Levine (1921) makes the following statement in regard to these tests.

A very serious objection to such classifications as those of MacConkey, Bergey and Deehan, and Jackson is their extreme flexibility and complexity, for as the number of fermentable substances or other characters observed increases, the number of "varieties" increases geometrically (approaching infinity) and soon produces a most unwieldy scheme.

For this reason and because the fermentable characteristics of the coli-aerogenes group seem to vary upon dissociation of these organisms we have not used any of these rare sugars. While they are interesting and no doubt important for some purposes they seem to be of little value in interpreting water quality.

Koser (1925) studied the utilization of the salts of other organic acids similar to citric acid by the coli-aerogenes group. He decided that none of them afforded the same distinction between the intestinal *Bact. coli* and other members of the group that citric acid salts did. Though both the uric acid and the citrate tests are recommended in Standard Methods these two tests are very similar and the citrate test seems to be the more reliable. We therefore selected Koser's citrate test in preference to the uric

acid test.   We have also included the methyl-red test, the Voges-Proskauer reaction and the indol test for reasons that will appear later.

The methyl-red test has been and probably still is the most popular and widely used differential test in water laboratories. The test was introduced by Clarke and Lubs and is practically the same today as when originated.   It depends upon the ability of the aerogenes section to attack the acids produced in their primary fermentation of glucose broth and raise the pH of a properly buffered medium and the inability of the coli section to utilize their acid end products in this fashion.   It is therefore essentially a pH determination with methyl-red as an indicator in a special medium.   Its success depends upon a sufficiently long incubation period to allow the differences in the complete carbohydrate metabolism of the *Bact. coli* and *Bact. aerogenes* organisms to take place.

This test is very valuable but has its limitations.   Other organisms often associated with the coli-aerogenes group in water and sometimes found as contaminants of coli-aerogenes group colonies on streaked isolation plates, such as the *Cl. Welchii* group, also produce methyl-red positive tests like those of *Bact. coli*. Some soil organisms have the ability to attack organic acids in a way similar to *Bact. aerogenes* and give methyl-red negative results.   Organisms such as those mentioned above, either alone or with members of the coli-aerogenes group, may produce erroneous methyl-red tests.   It is also apparent that mixtures of the coli and aerogenes sections can only give one reaction.   The mere presence of *Bact. coli* at the start does not assure a methyl-red positive in such cases.   The result will depend upon the factors discussed in the first section of this paper.   In this case, as in preliminary enrichment, the result is unpredictable.   The ratio of the two strains present at the start, their lag phases, maximum growth rates and sensitivity to the products of metabolism all affect the result.   In general, the shorter the incubation period, with twenty four hours as a limit, the greater is the chance for a methyl-red

positive result with coli-aerogenes mixtures. Ruchhoft, Kallas and Chinn (1931) have shown that when *Bact. coli* and *Bact. aerogenes* mixtures were planted together into glucose peptone-phosphate broth the proportion of methyl-red positives decreased as the period of incubation at 37°C. was increased from one to five days. After four days (the incubation period recommended by standard methods (1925)) only 74 per cent of the mixed *coli-aerogenes* cultures indicated *Bact. coli* by a methyl-red positive result while *Bact. coli* were recovered from 95 per cent of them. With pure *Bact. coli* strains methyl-red positive results will be obtained if the test is made at any time after the first twenty-four hour incubation period.   There is however, a considerable variation in ability of different *Bact. aerogenes* strains to utilize the organic acids formed during the primary fermentation.   This is shown by the different times required for various *Bact. aerogenes* strains to produce methyl-red negative tests.

The pH curves of twenty successive daily transplants into lactose broth of 2 different aerogenes strains was followed for ten days.   These pH curves for the two strains were different on the initial cultures and indicated that the first one had the greater ability to utilize acid and increase the pH.   These characteristic pH curves for the two strains continued throughout the experiment, the final curves for each strain being practically duplicates of the first and showing the same characteristic difference in the velocity at which the pH was increased.   This experiment indicated the constancy of the fermentative reactions produced by these strains.   A similar experiment on *Bact. coli* strains indicated a similar constancy and the lack of ability to utilize the organic acids.   Some aerogenes strains produce methyl-red negative tests in twenty-four hours while others require up to three days or more to accomplish the same result.   Methyl-red positive tests may be obtained with some pure aerogenes strains after forty-eight hours and methyl-red neutral or doubtful tests may even be obtained with such strains after seventy-two hours, and occasionally longer, at 37°C.   Some investigators have considered cultures producing neutral methyl-red tests with positive Voges-Proskauer reactions as anomalous or intermediate

forms in the group. We have however found this variability in the methyl-red reaction among aerogenes strains that were regular in their inability to produce indol and ability to utilize citrate. We therefore regard such strains as normal members of an aerogenes section that has considerable variation in the organic acid utilizing velocity of the various strains.

The character of the methyl-red test and the variation in the ability of the aerogenes strains to utilize the organic acids justifies the rather long incubation period of four days at 37°C. suggested by Standard Methods. Levine (1921) believes that the incubation period necessary for an accurate differentiation with the methyl-red test is too long for a good routine test. Prescott and Winslow, (1924) considered the procedure recommended by the 1923 Standard Methods as needlessly complicated and state that:

A single additional subculture in dextrose peptone phosphate broth for the methyl-red test would seem to be sufficient for all practical purposes; and we believe that such a test should be made where full knowledge of the sanitary quality of a water sample is desirable.

The data presented by several investigators which we will discuss later indicates that the results of the methyl-red test alone are not entirely reliable. In conjunction with other tests it is very valuable and therefore we have selected it as one of our tests.

THE VOGES-PROSKAUER TEST

The Voges-Proskauer test is also widely used in water laboratories. The reaction depends upon the ability of *Bact. aerogenes* to produce acetyl-methyl-carbinol in a peptone glucose broth with a resultant color reaction upon the addition of an alkali. Levine (1921) selected this test as the most satisfactory for routine work on account of its simplicity and the fact that the reaction could be obtained within twenty-four hours at 37°C. Chen and Rettger (1920) also have found it satisfactory and constant. It has recently been severely criticized. Several water analysts have reported it to be of no value. Linton (1924) first reported anomalous reactions with the Voges-Proskauer test and reported that acetyl-methyl-carbinol was a transient end product of glucose fer-

mentation. Paine (1927) reported the destruction of the acetyl-methyl-carbinol by certain organisms in the coli-aerogenes group. Williams and Morrow (1928) confirmed Paine's work and reported that aerobic spore forming organisms and the green fluoresent bacteria besides certain strains of *Bact. coli* and *Bact. aerogenes* could destroy acetyl-methyl-carbinol. Ruchhoft, Kallas and Chinn (1931) also reported that mixtures of *Bact. coli.* and *Bact aerogenes* could not be depended upon to exhibit the presence of *Bact. aerogenes* by the Voges-Proskauer reaction with incubation periods between one and five days at 37°C.

It must also be remembered that other organisms, particularly soil forms which may be present as contaminants of the coli-aerogenes group also give the Voges-Proskauer reaction. Such organisms have been reported by Meyer (1918), Ginter(1927) and Koser and Shinn (1927).

After a series of comparative tests we found the Digestive Ferments Company's dehydrated M.R.-V.P. medium more uniform and superior to the glucose-peptone-phosphate broth prepared in our laboratory for the Voges-Proskauer reaction. We have adopted this dehydrated medium. In making the test an equal volume of 10 per cent KOH solution is added to the cultures as recommended by Standard Methods. We have found the Standard Methods recommendation, to leave the cultures stand overnight at room temperature before making readings, unsatisfactory; and believe that some method to hasten the reaction so that the observation time can be shortened is necessary. Bedford (1929) suggested the use of a small amount (10 mgm. to 2.5 cc. of culture) of sodium peroxide before adding the KOH. This is very successful in hastening the color reaction. For routine work, however, we prefer incubating the cultures at 37° or 45°C. after the addition of KOH and making color observations after one, two and six hours. We obtain the majority of the positive reactions in less than one hour by this procedure. Recently Werkman (1930) reviewed the various methods of hastening the color production and the chemistry of the reaction. He suggests a new method of hastening the reaction which appears to be very satisfactory. Werkman's method depends upon the rapid

oxidation of acetyl-methyl-carbinol to diacetyl by the addition of ferric chloride as a catalyst. The diacetyl reacts with the peptone in a alkaline solution to produce a copper coloration. Two drops of a 2 per cent ferric hydroxide solution are added to 5 cc. of culture. Then the NaOH or KOH is added as in the usual procedure and the tube shaken. The copper color indicating a positive result usually develops within one hour. We have found that returning the tubes treated in this way to the 37°C. incubator also hastens the color formation so that the majority of the positive tests are obtained in fifteen to thirty minutes. We believe that this method has advantages and is desirable. Werkman reports that it has produced positive results when the "standard" procedure has failed to detect acetyl-methyl-carbinol. We, however, have never obtained positive reactions with Werkman's technique when such reactions were not also obtained with our regular procedure.

We have undertaken a number of experiments on some of the vagaries of the Voges-Proskauer reaction. Data have been collected on the following points:

1. The time required for the reaction and the possibilities of self destruction of the acetyl-methyl-carbinol with pure *Bacterium aerogenes* cultures isolated from water.

2. The possibility of acety-methyl-carbinol production by members of the *Bacterium coli* section.

3. The destruction of acetyl-methyl-carbinol by *Bact. coli*.

In our study of the first problem, each of 30 pure aerogenes cultures was planted into 7 tubes of the M.R.-V.P. medium and incubated at 37°C. The Voges-Proskauer tests were made on one series of cultures after various incubation periods of from one to seven days with the following results:

TOTAL NUMBER OF STRAINS	CLASS 1— NUMBER GIVING POSITIVE V.P. TEST EVERY DAY	CLASS 2— NUMBER GIVING − TEST ON FIRST DAY BUT + THEREAFTER	CLASS 3— NUMBER GIVING + TEST ON FIRST DAY BUT − THEREAFTER	CLASS 4— NUMBER GIVING + TEST FOR 2 DAYS BUT − THEREAFTER	CLASS 5— NUMBER GIVING + TEST FOR 3 DAYS BUT − THEREAFTER	TOTAL NUMBER NOT GIVING + TEST ON EVERY DAY
30	21	3	3	2	1	9

The nine cultures which did not give positive tests every day were repurified and the experiment was repeated. The three cultures in class 2 then gave positive tests every day. With the remaining cultures the results were very much as they were in the first experiment. However, three colonies were picked from strain number 23 which was in class 3, and one of these subcultures continued to give the reaction throughout the experiment. One of the other subcultures failed to give the reaction after one day as in the first experiment while the other one continued to give it for three days.

This experiment confirms the results of Linton (1924) and Georgia and Morales (1926) who reported more frequent negative V.P. results with incubation periods over three days. It confirms the work of Paine (1927) and Williams and Morrow (1928 that *Bact. aerogenes* may be self destructive of acetyl-methyl-carbinol. It also shows that it is impossible to choose a definite incubation time with the assurance that a positive V.P. reaction will be obtained for every *Bact. aerogenes* culture.

PRODUCTION OF ACETYL-METHYL-CARBINOL BY BACT. COLI

To determine the possibility of acetyl-methyl-carbinol production by *Bact. coli* 39 strains have been tested at least 20 times, during the past year, after two- and three-day incubation periods at 37°C. in the M.R.-V.P. medium with negative results. These cultures were also tested after eighteen and twenty-four hours incubation with negative results. More highly buffered media were therefore prepared by adding 2, 4, 8 and 16 grams per liter of K_2HPO_4 to the standard medium.

Five *Bact. coli* strains were planted into these with two *Bact. aerogenes* controls. The pH of all cultures was followed and the Voges-Proskauer tests were made after one, two, three, five and ten days at 37°C. The *Bact. aerogenes* produced positive reactions in all of these media except the most highly buffered one, but not a single instance of acetyl-methyl-carbinol production was obtained with any of the *Bact. coli*, either with our regular technique or Werkman's. We believe that the possibility of acetyl-methyl-carbinol production by the *Bact. coli* section as

intimated by Kluyver and Donker (1926) is very remote.[2]   However, the reversion of certain soil forms from M.R. + V.P. − upon isolation to M.R. − and V.P. + after several weeks cultivation as described by Koser (1924) may be interpreted as the production of acetyl-methyl-carbinol by *Bact. coli*. Speaking about such reversion Koser says:

> While it is realized that contamination cannot be positively excluded in these cases, nevertheless, it is believed that the change in the tests as observed cannot be accounted for in this manner. It seems more probable that as a result of laboratory cultivation the secondary or alkaline fermentation was "speeded up" until finally the reversion of reaction to give an alkaline test to methyl-red took place within the customary time of four days. The production of acetyl-methyl-carbinol, as shown by a positive Voges-Proskauer test was never apparent until this reversion had taken place.

Eight of Koser's cultures which exhibited such reversions in their methyl-red and Voges-Proskauer reactions belonged to · the aerogenes section when judged by their ability to utilize citrate and uric acid, their carbohydrate fermentations and Endo colonies and their inability to produce indol. We conclude therefore that with the standard Clark's glucose broth or the Digestive Ferments Company's M.R.-V.P. medium the possibility of acetyl-methyl-carbinol production by *Bact. coli* may be safely ignored in water analysis.

DESTRUCTION OF ACETYL-METHYL-CARBINOL BY BACT. COLI

Whether *Bact. coli* will affect the finding of acetyl-methyl-carbinol in mixed culture with *Bact. aerogenes* may depend upon several factors. It may depend upon the ability of the *Bact. coli* to destroy acetyl-methyl-carbinol after it has been produced, or it may depend upon the growth rates of the two organisms in Clark's medium and the ascendency of the *Bact. coli* and its products of metabolism in sufficient quantity to prevent the proper secondary metabolism of the *Bact. aerogenes* with its accompanying acetyl-methyl-carbinol production.

[2] In a recent letter from Dr. Kluyver he said that the statement regarding the production of acetyl-methyl-carbinol by *Bact. coli* was based on one apparently successful result in 1926. Since that time they have repeated their experiments without success. He has not had the opportunity to withdraw the original statement since and asked us to explain this situation for him.

To determine the ability of *Bact. coli* to destroy acetyl-methyl-carbinol an acetyl-methyl-carbinol medium was produced by growing a *Bact. aerogenes* strain in glucose broth for forty-eight hours at 37°C. This culture which contained acetyl-methyl-carbinol was divided into three portions of about 1 liter each.[3] The first portion was tubed and sterlized and contained the killed cells of *Bact. aerogenes*. The *Bact. aerogenes* cells were filtered out of the other two portions and one half of 1 per cent of dehydrated M.R.-V.P. medium was added to the third portion. These three different acetyl-methyl-carbinol media were tubed and sterilized. Thirty-eight *Bact. coli* strains were planted into tubes of each medium. The cultures and control tubes were incubated at 37°C. In the first two media, where additional M.R.-V.P. medium had not been added, all cultures remained methyl-red negative during the entire experiment and there was destruction of acetyl-methyl-carbinol in only 2 cases in each medium during ten days. In the filtered medium series an excellent growth of 100,000,000 or more *Bact. coli* per cubic centimeter was obtained with each of the 38 strains. This proved that the failure of more strains to destroy acetyl-methyl-carbinol in the first and second series was not due to the failure of *Bact. coli* to grow in the medium.

The results obtained with these *Bact. coli* strains in the acetyl-methyl-carbinol medium to which additional M.R.-V.P. medium has been added were as follows:

	INCUBATION TIME				
	Initial	2 days	4 days	6 days	10 days
Total number of strains.....................	38	38	38	38	38
Number of M.R. − V.P. +.................	38	0	12	14	14
Number of M.R. ± V.P. +.................		4	4	3	0
Number of M.R. + V.P. +.................		34	22	20	18
Number of M.R. + V.P. ±.................				0	2
Number of M.R. − V.P. −.................				1	4

[3] Contrary to some of the published reports it was found that acetyl-methyl-carbinol was volatile. Portions of this medium were steam distilled and tests for acetyl-methyl-carbinol were obtained on the various fractions following the addition of peptone and potassium hydroxide.

Bact. aerogenes cultures that destroy acetyl methyl-carbinol destroyed it in all of these media in four to six days.   One *Bact. coli* strain No. 32 also slowly destroyed acetyl-methyl-carbinol in all of these media.   The experiments showed however, that few *Bact. coli* have this ability and even when they have, the destruction requires more than four days   We must conclude therefore that the actual destruction of acetyl-methyl-carbinol by *Bact. coli* is rarely the cause for the vagaries of the reaction in mixed *Bact. aerogenes* cultures.

The possibility of failure to obtain the acetyl methyl-carbinol from *Bact. aerogenes* cultures due to overgrowth of *Bact. coli* is governed by factors which have been discussed in the first section of this paper.   The initial ratio of the strains present and their lag phases will affect the result.   The growth rates and the effect of the products of metabolism on the strains involved will also affect the result.   It is therefore impossible to predict how frequently the acetyl-methyl-carbinol reaction will fail to be obtained with contaminated *Bact. aerogenes* cultures without knowing the exact conditions involved in each case.   Experiments have shown, however, that this reaction may be easily interferred with and positive tests will not be obtained on nearly all occasions when *Bact. aerogenes* are present in the culture.   Implantation of *Bact. aerogenes* strains into twenty-four-hour *Bact. coli* cultures in M.R.-V.P. medium showed us that under these conditions the V.P. reaction is rarely obtained.

From the foregoing study of the possibilities of interference with the Voges-Proskauer reaction, the vagaries of the test often reported in routine water work are not surprising.   The purity of the cultures, the length and temperature of incubation, and the technique for hastening the reaction or making the readings all had an effect on the final result.   It is therefore interesting to compare the results obtained in four laboratories with this test as shown in table 10.   In view of the well known fact that the *Bact. aerogenes* have a lower death rate in streams and stored waters than the *Bact. coli* the rather low percentage of Voges-Proskauer positive reactions reported by all laboratories is rather surprising. Direct counts on our waters have indicated a much higher average

proportion of *Bact. aerogenes* than are indicated by the Voges-Proskauer tests. Our growth rate studies have not indicated that the *Bact. aerogenes* are outgrown by the *Bact. coli* during preliminary enrichment. Still, the table shows very low Voges-Proskauer results from four widely separated laboratories. Interference with the reaction by the various causes already mentioned is we believe the explanation for these low results. It will be noted that the proportion of positive Voges-Proskauer tests was almost doubled in our laboratory from 1928 and 1929 to 1930. We do not ascribe this to a real difference in the proportion of *Bact. aerogenes* cultures isolated. We believe it is the result of an improved medium, a shortening of the incubation period and an improved technique.

We therefore conclude that, due to the high incidence of contaminated cultures and irregularities of the *Bact. aerogenes* strains, the Voges-Proskauer test is not of much value as carried out at present in water laboratories. If it is to be made at all on routine cultures it should be repeated at least twice after various incubation periods such as one and three days. Its correlation with the methyl-red-test for reasons shown is one of its weak points in routine work. For this reason the results of the methyl-red and Voges-Proskauer reactions are often misleading. These two tests should not be used alone in water analysis. When they are used with the other two tests to be described, however, they are of some value.

KOSER'S SODIUM CITRATE TEST

Koser (1923) first studied the ability of organisms of the coli-aerogenes group to utilize salts of citric acid as the only source of carbon. He made a very careful study of strains obtained from animal feces and soils and discovered that the methyl-red positive, Voges-Proskauer negative section of the coli-aerogenes group could be separated into two divisions, dependent upon their ability to utilize sodium or potassium citrate. Fecal methyl-red positive, Voges-Proskauer negative strains were unable to utilize citrates. Soil methyl-red positive, Voges-Proskauer negative cultures were able to utilize citrates.

The following table illustrates the significance of Koser's findings:

| SOURCE OF CULTURE | REACTIONS | | | E.M.B. COLONY TYPE |
	M.R.	V.P.	Citrate	
Soil..............................	+	−	+	Coli or aerogenes type (see fig. 2)
	−	+	+	Aerogenes type
Human and animal feces..............	+	−	−	Coli type

In other words there were both M.R. positive, V.P. negative organisms and M.R. negative, V.P. positive organisms in soil which were alike in their ability to utilize citrate. The ability of the organisms to utilize salts of citric acid serves therefore as an index of their probable source superior to methyl-red and Voges-Proskauer tests.

The value of this test was soon recognized and it was included among the recommended differential tests in the Standard Methods (1925). Since the test was comparatively new no statement regarding the interpretation of the test was included in the 1925 edition of Standard Methods. This led to confusion in interpretation of the results obtained with it in water laboratories. Bardsley (1926) in England used this test in conjunction with the M.R., V.P. and indol tests and, apparently, because it failed to correlate as expected with these tests, dropped the citrate test as the least valuable. Antithetical opinions are found in the American literature. One investigator relied solely on the "Citrate" test in studying the incidence of fecal *Bact. coli* strains. This, of course, is poor practice because growth in citrate solution proves the presence of *Bact. aerogenes* or other citrate utilizers but it does not prove that *Bact. coli* are not also present.

Simmons (1926) and Lewis and Pittman (1928) modified the original Koser's medium, principally by the addition of agar so that the test can be made on streaked plates. Tonney and Noble (1930) confirmed 1919 cultures in both Lewis and Pittman's ferric-ammonium citrate agar and in Koser's sodium citrate solution. Of these 1919 cultures, 630 were reported as citrate utilizers in Koser's medium while only 352 were reported as

citrate utilizers on solid ferric-ammonium citrate agar. This shows that the liquid medium is superior to the solid medium in the detection of citrate utilization. Our studies of these media have confirmed the above results. We, therefore, prefer, and use, the liquid medium as described in standard methods for routine tests.

The Koser's citrate medium has characteristic advantages and disadvantages that do not appear to have been sufficiently emphasized. We will therefore discuss briefly the following points which we have learned in the course of some 9000 citrate tests made during the past year.

1. Growth of the coli-aerogenes group in the solution.

2. Effects of products of metabolism and viability of cultures.

3. Deoxygenation of the medium as a criterion of difference between the two sections of the group.

4. Changes in the pH of the medium by the coli-aerogenes group.

5. Possibility of purification by use of the medium.

GROWTH OF THE COLI-AEROGENES GROUP IN KOSER'S CITRATE SOLUTION

Our studies have indicated that all members of the coli-aerogenes group multiply in the Koser's citrate medium. The *Bact. coli* section, as reported by Koser (1923), is unable to produce a visible growth or turbidity in the medium within five to seven days at 37°C. This has been observed on more than 2000 cultures during the last two years. Thirty-eight *Bact. coli* strains isolated from water were transfered to Koser's medium and observations were made daily for five days at 37°C. and thereafter weekly for nine weeks at room temperature. At the end of one week no cultures had visible growth. After two weeks 3 strains still had no evidence of growth. The remainder of the cultures were also clear but contained slight sedimentary deposits of bacterial cells. If these tubes were shaken the sediment was dispersed and a slight turbidity appeared. Such slight turbidity, noticed only after shaking and after a week or more of incubation, is typical of the growth of *Bact. coli* and some other organisms in citrate

solution. Such growth may be confused by some with *Bact. aerogenes* but we have always found that the *Bact. aerogenes* section will produce a turbidity which is visible without shaking after shorter incubation periods. Of the 38 *Bact. coli* strains described above that were held for nine weeks, only one produced a turbidity that was visible before the tube was shaken to disperse the sediment. At the end of the experiment all of these *Bact. coli* cultures produced the same type of colonies on E.M.B. agar as when they were transferred into Koser's citrate medium. A colony from each strain that had been cultivated for sixty-three days in the citrate medium was picked and differential tests were made. All cultures had the same differential characteristics as they initially had, and were unable to produce any visible turbidity (that could be observed even after shaking the tube) after our customary incubation period of three days at 37°C. We have found that *Bact. coli* innoculums as low as one organism per cubic centimeter multiply quite rapidly at 37°C. in Koser's citrate medium until population levels of from 1 to 5 million per cubic centimeter are reached. Such population levels are often produced in twenty-four hours but are not sufficient to produce visible growth. Thereafter there seems to be very little change in the population level of pure *Bact. coli* strains. The products of bacterial metabolism in citrate medium do not seem to be of sufficient quantity, or are not sufficiently toxic, to produce any effect on the viability of *Bact. coli* strains regardless of the length of the incubation period. Therefore we have adopted the practice of holding the citrate tube culture for a week or two in routine differential studies to recover the strain if further study is necessary, regardless of whether there is visible growth. We have never lost a *Bact. coli* strain by this procedure. In fact we have recovered *Bact. coli* strains from Koser's citrate medium cultures one year old which had stood at laboratory temperatures and in which the original volume of the medium had been reduced 75 per cent or more by evaporation. These cultures were streaked on E.M.B. agar plates and usually typical colonies were obtained but in some cases rough form colony *Bact. coli* dissociants were observed.

GROWTH OF BACTERIUM AEROGENES SECTION IN KOSER'S CITRATE MEDIUM

The *Bact. aerogenes* section of the group grow more rapidly and attain higher population levels than the *Bact. coli* section. Populations of 25 to 150 million bacteria per cubic centimeter are usually obtained in twenty-four hours at 37°C. Thereafter, there is little change in the population in the ordinary cotton plugged culture tubes. Such populations are easily visible as a turbidity throughout the tube. A record was kept of the time required for 289 *Bact. aerogenes* strains to produce a turbidity throughout the citrate medium. Of these, 270 or 93.5 per cent gave visible growth in twenty-four hours at 37°C., 11 or 4 per cent after forty-eight hours and the remaining 8 or 2.5 per cent in seventy-two hours.

Tests by the dilution method, using lactose broth, tryptophane broth and citrate medium, showed that the latter medium was practically as sensitive in producing growth from very small innoculums of *Bact. aerogenes* as the two former. This confirms Koser's report (1923) on the reproduction sensitivity of the medium for *Bact. aerogenes*. We noted, however, that with such small innoculums the period required to produce a distinctly visible growth was sometimes lengthened to between twenty-four and forty-eight hours where larger innoculums produced visible growth in twenty-four hours. In routine differential work, however, the innoculum is usually large when the strain happens to be pure *Bact. aerogenes*. When the culture or colony picked contains principally *Bact. coli*, with *Bact. aerogenes* as contaminants, the innoculum may contain only a few aerogenes and it is these cases which sometimes only produce visible growth after forty-eight hours in the citrate medium.

Our routine cultures were formerly incubated for five days but the incidence of additional positive growth cultures between the third and fifth days was so low that we believe three days is long enough for routine work. We have noted that *Bact. aerogenes* can be transferred daily with a needle from tube to tube of Koser's citrate up to 15 transfers (length of our experiment) and positive growth will always be obtained in twenty-four, and at most forty-eight hours. The same treatment with *Bact. coli* cultures

never produces visible growth within three days though viable organisms are found after one day incubation of the fifteenth transfer. We have obtained routine cultures, however, which upon three days incubation produced very slight turbidities by dispersion of sedimentary cells when shaken. When such cultures were transferred to other citrate tubes no evidences of turbidity occurred in the same incubation time. Recovery of the organism by streaking on E.M.B. agar and differentiation of fished colonies showed apparently pure *Bact. coli*. It is probable that the original growth was due to another organism that was lost during this process. It may also be well to point out here that many of the positive growth cultures obtained in citrate medium in three days may be due to soil forms that are capable of utilizing citrate and are not members of the group but are often found with the coli-aerogenes forms.

EFFECTS OF PRODUCTS OF METABOLISM AND VIABILITY OF CULTURES IN KOSER'S CITRATE SOLUTION

One important characteristic of this medium is the apparent lack of effect of the products of metabolism of one strain of aerobic organisms upon any other aerobic organisms that are growing in it at the same time. This seems to be true for mixtures of *Bact. coli* and *Bact. aerogenes* strains, for *Bact. coli* and the extraneous soil forms utilizing citrate and for other groups. Unfortunately we have not studied the fate of Cl. Welchii group organisms when they are introduced into the medium mixed with *Bact. coli*. We know, however, that the *Bact. coli* reduce the dissolved oxygen content of this medium very little in cotton stoppered tubes.

When *Bact. coli* and *Bact. aerogenes* are grown together in Koser's citrate medium both strains can usually be recovered even after very long incubation periods. Sixteen different mixtures of *Bact. coli* and *Bact. aerogenes* strains were introduced into Koser's citrate medium and both strains were recovered from all mixtures at weekly intervals for five weeks. Eight of these mixtures were examined again after standing eleven months at room temperature and both strains were found in seven of them while in the eighth only *Bact. aerogenes* was recovered. These

and many other experiments all indicated the remarkable viability of both sections of the coli-aerogenes group in Koser's citrate solution. All the experiments so far described show that *Bact. aerogenes* multiplies easily rapidly producing visible growths while *Bact. coli* grows more slowly and never reaches visible population levels in the citrate medium. Unfavorable conditions are not produced in the medium by either section of the group which prevent the multiplication of the section initially in the minority to its normal population level. This makes the detection of *Bact. aerogenes* present as contaminants of *Bact. coli* easy and certain in citrate solution while it is difficult and uncertain to detect such contaminants by means of the V.P. test in Clark's broth.

DEOXYGENATION AND pH CHANGES IN KOSER'S CITRATE SOLUTION

The production of visible growth is not the only criterion distinguishing the *Bact. coli* and *Bact. aerogenes* sections of the group when grown in citrate medium. Two other changes take place in this medium which may be used to differentiate these sections. These changes are the rate of abstraction of dissolved oxygen from the medium and the changes in hydrogen ion concentration in the medium.

When *Bact. aerogenes* is grown in sodium citrate medium in completely filled glass stoppered bottles complete depletion of the dissolved oxygen in the medium usually takes place in twenty-four hours at 37°C., while with the *Bact. coli* section there is but slight reduction of the dissolved oxygen during the same time. Details of these experiments cannot be given in this paper. While the method is rather impractical for routine purposes it seems to be capable of showing slight differences in the citrate utilization ability of the coli-aerogenes group strains.

The pH of Koser's citrate medium cultures may be followed more easily than the dissolved oxygen content. Using the method of Hurwitz and Kraus (1929) we have made pH determinations on a number of coli-aerogenes group strains with the results shown in table 2. This checks Koser's (1923) results and shows that the pH resulting from a three or four days' growth of these

TABLE 1

Comparison of results of Voges-Proskauer tests on cultures from water by several observers employing various techniques

	OBSERVER				
	Bahlman and Sohn (1924)	Bardsley (1926)	Lewis and Pittman (1928)	(S.D.C.) Kallas, Weed, Berberich and Clark (1928) (1929)	(S.D.C.) Kallas, Chinn, Coulter and Luthardt (1930)
Type of water.............	Ohio River: raw, settled, filtered and chlorinated	Potable and polluted	Polluted and high sanitary quality waters	Lake Michigan raw and chlorinated	Lake Michigan raw and chlorinated
Medium..................	Clark's prepared in laboratory	Clark's prepared in laboratory	Clark's prepared in laboratory	Clark's prepared in laboratory	Clark's Difco M.R.V.P.
Incubation temperature....	30°C.	30°C.	30°C.	37°C.	37°C.
Incubation period.........	5 days	3–5 days	5 days	4 days	3 days
Treatment previous to reading......	37°C. for 8 hours		Chen andRettger, 30°C. for 3 hours	Standard Methods, 1925	37°C. for 1 to 6 hours
Total cultures............	1,223	1,441	320	1,365	1,022
Number of V.P. positive.....	341	180	53	203	310
Per cent V.P. positive.......	27.8	12.5	16.6	14 8	30 6

TABLE 2

pH obtained in Koser's citrate medium using 38 Bact. coli and 26 Bact. aerogenes strains

	INCUBATION PERIOD				
	1 day	2 days	3 days	4 days	6 days
Average *Bact. coli*.....................	6.82	6.75	6.74	6.60	6.80
Average *Bact. aerogenes*................	7.43	7.60	7.70	7.80	8.23
Maximum *Bact. coli*....................	6.90	6.90	6 80	6.60	6.80
Maximum *Bact. aerogenes*..............	7 80	7.80	7.90	8.20	8.40
Minimum *Bact. coli*....................	6 80	6 60	6.70	6.60	6 80
Minimum *Bact. aerogenes*..............	7 00	7 40	7.50	7.60	8.10
Maximum *Bact. coli*..................	6.90	6.90	6.80	6 60	6.80
Minimum *Bact. aerogenes*.............	7 00	7.40	7.50	7.60	8.10

TABLE 3

Incidence of citrate utilizing organisms from various sources (determined in Koser's citrate medium)

	SOURCE							
	Human and animal feces		Polluted water		High sanitary quality water		Soils or cereals	
	Cultures tested	Citrate +	Cultures tested	Citrate +	Cultures tested	Citrate +	Cultures tested	Citrate +
Koser (1923) (1924)...........	118	11	107	38	90	75	72	70
Pawan (1925)..................	432	16	210	18	240	195	214	193
Bardsley (1926)			979	140				
Raghavachari (1926)....			1,074	331	500*	248*		
Lewis and Pittman (1928).......			83	22	237	156		
Tonney and Noble (1930).......	1,256	162	377	249			286	229
Houston (1928).................	92	0						
Hicks (1927).............	150	16						
Brown and Skinner (1930)......			153	50				
Sanitary District of Chicago (1930)........................	486	28	2,872	1,575				
Total of above...............	2,534	233	5,855	2,423	567	426	572	492
Per cent citrate +...........		9.2		41..5		75.0		86.0

* Filtered water samples.   These are not included in the high sanitary quality water total.

organisms in citrate medium is also sufficient to differentiate them. The pH changes induced by the M.R. positive, V.P. negative, citrate positive soil forms described by Koser were also determined. Six such strains were used and the results obtained were similar to those for the *Bact. aerogenes* group given in table 2.

Before concluding our study of the citrate medium we wish to point out its possible use in purification of mixed coli-aerogenes group cultures. Due to the lower population levels maintained in citrate medium streaked plates from the medium usually produce well distributed isolated colonies. Both *Bact. coli* and *Bact. aerogenes* will be produced on such plates from mixed cultures and complete separations can be more easily made than from plates streaked with cultures from media maintaining higher population levels.

Some results that have been obtained with the Koser's citrate on coli-aerogenes group isolations are summarized in table 3. The scarcity of citrate utilizing organisms in human and animal feces is strikingly shown. The greatest variation in percentages of citrate utilizers from polluted waters is illustrated in the results of Bardsley and Tonney and Noble. Bardsley found scarcely any more citrate utilizers in polluted water than are usually reported in feces. Tonney and Noble found the percentage of citrate utilizers almost as great in surface water and sewage as is usually reported in waters of high sanitary quality. Our results on polluted water indicate that at times there might be considerable variation in the percentage of citrate positive organisms isolated. The fact that there is no great difference in the percentage of citrate positive organisms isolated from raw sewage and from surface waters not highly polluted decreases the value of the test as a criterion of pollution. We found that the incidence of citrate utilizing organisms is usually higher in cultures which are reported as mixed, with or without spores, by Gram stain examination. The fact that the citrate test is a negative test for pollution and does not show that organisms of fecal origin are not also present decreases the value of the test alone. It is valuable, however, as an aid in the classification of strains and also as an index of probable mixed strains. It is our conclusion, therefore, that this

test should not be used alone but should be used in conjunction with other tests to be of the greatest value.

THE INDOL TEST

We did not adopt the indol test until a study of more than 1,000 cultures with the three tests already described had convinced us that more information was necessary for an intelligent interpretation of a great many of the results. This test was formerly considered one of the best for the identification of fecal organisms and was required for the identification of the group in the (1905) edition of the Standard Method. Castellani and Chambers (1918) used the indol reaction as a basis of classification of the Genus Escherichia and reported 17 indol positive species and one negative species. Levine (1921) found that the indol reaction correlated well with fermentation in salicin but not with dulcitol and glycerol. He used the M.R., V.P., and uric acid reactions and fermentation in sucrose, salicin and glycerol as a basis of classification of the coli-aerogenes group and did not use the indol reaction. Bergey (1925) reported that 16 of 22 species of the genus *Escherichia* and 2 out of 5 species of the genus *Aerobacter* produce indol. Jordan (1928) stated that *Bact. aerogenes* regardless of origin may produce indol. Blumenberg (1928) reported that indol production is not a constant characteristic of *Bact. coli* and that when used as a test for fecal contamination pseudo negatives may be obtained. He also reported that *Bact. cloacae* may produce indol and give pseudo positives. Nevertheless the indol test is still used and considered important by Houston (1928). Bahlman and Sohn (1924), Bardsley (1926) and Lewis and Pittman (1929) all used the indol test in conjunction with other tests with interesting results which we will discuss later.

Perry (1929) concluded that indol, methyl-red, citrate and cellibiose were the best differential tests. Hicks (1927) in Shanghai used the same tests we have used on organisms from human and animal feces and from soil. He concluded that the indol and citrate tests were of value but that the methyl-red and Voges-Proskauer tests were of no value. Raghavachari

(1926) also used these four tests and reported a very high degree of inverse correlation between the indol and citrate tests. Our experience in regard to the indol test is in accord with the results of Hicks and Raghavachari. We shall show that the indol test due to certain characteristics is very useful especially when used in conjunction with the citrate, methyl-red and Voges-Proskauer tests.

TECHNIQUE OF INDOL TEST

Kovács (1928) described a modified technique for Ehrlich's indol test which we have found to be the best of any that we have yet tried. The reagent for the test contains 5 grams of *p*-dimethyl-amino benzaldehyde, 75 cc. of amyl alcohol and 25 cc. of concentrated hydrochloric acid. We have had the best success with Eastman's C.P. *p*-dimethyl-amino benzaldehyde and with Merck's laboratory reagent amyl alcohol. Some brands of C.P. amyl alcohol are not satisfactory, because they apparently contain furfurals which produce a dark color reaction with the *p*-dimethyl-amino-benzaldehyde. This reagent should have a yellow or light brown color when made up. This test depends upon the extraction of the indol in the culture by the amyl alcohol. The indol reacts with the *p*-dimethyl-amino benzaldehyde in the surface layer of the amyl alcohol so that the color reaction is not affected by the color of the medium.

We have found the Digestive Ferments Company's dehydrated tryptophane broth excellent for the indol test and use this medium dispensed in tubes in 4 cc. amounts for the test in our laboratory. The test is made after a twenty- to twenty-four-hour incubation period at 37°C. We have found that the addition of potassium persulphate and the 3 cc. of the *p*-dimethyl amino benzaldehyde reagent as recommended by Kovács was not necessary. We therefore omit the potassium persulphate entirely. We simply add from 0.3 to 0.4 cc. of Kovács amyl alcohol indol reagent to each culture, shake the tube let it stand for a minute and make the readings. We have made over 11,000 tests by this method in our laboratory during the past year with very excellent results.

SENSITIVITY OF INDOL TEST

To show the sensitivity of this indol test and the lack of interference with it by other organisms we wish to describe briefly several experiments. Thirty-eight *Bact. coli* strains (in which there were members of the following species according to Levine's classification *Bact. coli, Bact. acidi-lactici, Bact. neapolitanum* and *Bact. communior*) were used in studying the indol test. Of these, 24 gave positive indol tests after six hours incubation by the technique described above. After eighteen hours they all gave positive tests. After twenty-four hours the minimum amount of culture required to give a positive indol test was determined. Thirty-five of these strains showed a positive indol reaction with 0.1 cc. of culture after twenty-four hours while the remaining three produced weak reactions with 0.1 cc. of culture. From the 4th to the twenty-fifth day all of these strains produced positive indol reactions when 0.1 cc. of culture was added to 0.2 cc. of the indol reagent. This experiment shows the speed at which indol is formed by *Bact. coli* in this culture medium, the sensitivity of this indol test and the stability of the indol when once formed. All of these *Bact. coli* strains have been held one year on agar slants, six months in tryptophane broth, nine weeks in Koser's sodium citrate solution, they also have been repeatedly recultivated in tryptophane broth, lactose broth, glucose (Clark's) broth, brilliant green bile broth, E.M.B. agar plates and Noble's ferrocyanide citrate agar plates and they have always produced indol when reinoculated into tryptophane broth. Representatives of the above cultures were also treated in various other ways but the ability to produce indol was constant. We must therefore conclude that the ability to produce indol is a very stable characteristic of *Bact. coli* strains that cannot be easily changed by laboratory treatment.

NON-INTERFERENCE OF OTHER ORGANISMS WITH THE INDOL TEST

Another valuable characteristic of the indol test is the fact that it is not interfered with by other organisms. We have shown that in the alkaline tryptophane broth indol was indicated by 0.1 cc. of culture even after twenty-five days incubation with

pure *Bact. coli* cultures.   Our studies indicate that with *Bact. aerogenes* and many other water organisms present with *Bact. coli* in tryptophane broth the indol produced will be present for similar lengths of time.   In other words when indol is once produced it is not easily broken down by other organisms and can be easily detected.   The only conceivable condition under which a negative indol test might be obtained with *Bact. coli* strains which produce indol would be when other organisms outgrow and produce conditions in the medium unfavorable for the growth of *Bact. coli*. The possibility of this condition occurring was investigated with *Bact. coli* and *Bact. aerogenes* mixtures.'   Seven representative *Bact. aerogenes* cultures were selected and each culture was inoculated into 15 tryptophane broth tubes which were incubated at 37°C. for twenty-four hours.   Good growth as shown by turbidity was obtained in each case.   Twelve culture tubes out of each series of 15 were then inoculated with 1 loop of 12 representative *Bact. coli* cultures.   A control tube of each *Bact. coli* strain was also planted and then all tubes were incubated for an additional twenty-four hours.   The entire set up then included 7 *Bact. aerogenes* strains of three tubes each for *Bact. aerogenes* controls, 84 different combinations of mixed *Bact. coli* and *Bact. aerogenes* cultures in which all of the *Bact. aerogenes* had a twenty-four-hour incubation period before the *Bact. coli* were introduced, and the 12 *Bact. coli* controls.   The indol test was made on all these cultures after the *Bact. aerogenes* had been incubated for forty-eight hours and the *Bact. coli* for twenty-four hours with the results shown below:

	ORGANISMS		
	Bact. coli	*Bact. aerogenes* and *Bact. coli* mixtures	*Bact. aerogenss*
Number of strains..........	12	84 different combinations	7 strains, 3 cultures of each
Incubation time............	24 hours	48 hours for *Bact. aerogenes*, *24 hours* Bact. coli	48 hours
Indol results...............	All +	79+, 3±, 2−	All −

All of the *Bact. coli* controls were indol positive and all *Bact. aerogenes* controls indol negative. Seventy-nine of the 84 mixtures produced strong indol reactions. Of the other five mixtures, which were all combinations of one *Bact. coli* strain No. 7 with the various *Bact. aerogenes*, 3 produced weak but positive indol tests and two were indol negative. We had learned from the indol sensitivity experiment that the No. 7 *Bact. coli* strain was the slowest indol producer. We therefore repeated the experiment using *Bact. coli* strain No. 7 with 26 *Bact. aerogenes* strains. This time the *Bact. aerogenes* cultures were planted and incubated for twenty-four hours before the No. 7 *Bact. coli* strain in one series and in another series of combinations one loop of the various *Bact. aerogenes* cultures and one loop of No. 7 *Bact. coli* were introduced at the same time. This *Bact. coli* strain failed to produce indol in 8 mixed cultures where the *Bact. aerogenes* strains had a twenty-four-hour longer period of incubation. In the remainder of this series of mixed cultures indol was produced in spite of the twenty-four-hour start of the *Bact. aerogenes* strains. In the series of mixed cultures where this slow indol-producing *Bact. coli* strain was planted at the same time as the various *Bact. aerogenes* strains indol was produced in every case in twenty-four hours. These experiments indicate that in almost all cases of mixtures of *Bact. coli* and *Bact. aerogenes* the presence of the *Bact. coli* strains will be indicated in twenty-four hours by indol formation in tryptophane broth. These experiments also show that indol production is a sensitive index of contamination of a *Bact. aerogenes* culture by *Bact. coli*. In this respect the test is very much superior to the methyl-red test for reasons already discussed.

FAILURE OF BACT. COLI TO PRODUCE INDOL

We believe that some of the reports of failure to produce indol by *Bact. coli* and of indol production by *Bact. aerogenes* are due to the confidence of the observers in colonial characteristics of the organism on isolation media. No doubt, even when the division is based on biochemical characteristics, some members of the *Bact. aerogenes* section produce indol and some members of the *Bact. coli* section do not. However, as shown by Koser

(1923), some soil forms which resemble *Bact. coli* on isolation media and are M.R.+ and V.P. – resemble aerogenes in their ability to utilize citrate and also in their inability to produce indol. It is their carbohydrate metabolism which apparently restricts their colony size and makes them resemble *Bact. coli* on isolation media, but as far as the above tests are concerned there is no more reason for calling them *Bact. coli* than *Bact. aerogenes*. Water analysts, however, on the basis of colony appearance and the M.R. and V.P. tests in the past, no doubt often reported such strains as *Bact. coli* which failed to produce indol. However, Koser has shown that this type is common to soils and rare in animal feces. Brown and Skinner (1930) reported a number of "*B. coli*" isolated from water which utilized citrate. They did not give the indol reactions of these cultures so it is possible that these were also soil forms, similar to those described by Koser, which may be classified as either coli-like or aerogenes-like, depending upon the reactions to which the greatest significance is attached by the observers.

We wish to point out here that among more than 2000 cultures that we have tested with the four tests described, the number that were indol − M.R. + V.P. − and citrate − were rare. We believe that pseudo negative indol tests from fecal *Bact. coli* trains are very seldom obtained.

INDOL PRODUCTION BY COLONY TYPES AND BACT. AEROGENES

If the various types of colonies observed on routine isolation plates are fished and examined for indol production the lack of confidence in the indol test (when isolated colonies are accepted as pure) is easily understood. The results obtained with 686 colonies fished from routine E.M.B. plates from Lake Michigan water samples are shown in table 4. This shows that the incidence of indol production decreased as the colony appearance varied from typical *Bact. coli* to typical *Bact. aerogenes*. If one considers each of these colony types as representative of definite coli-aerogenes strains then of course, indol production is a very variable characteristic of these organisms. We have shown in an earlier section of this paper that many of these colonies are mixtures

of *Bact. coli* and *Bact. aerogenes*. This explains the indol varia-
bility in such colonies. Bahlman and Sohn (1924) classified 1223
cultures from routine isolation plates from water samples accord-

TABLE 4

Indol production from various types of colonies on E.M.B. agar

TYPE OF COLONY	TYPICAL COLI WITH SHEEN	COLI TYPE WITHOUT SHEEN	BLUE COLI TYPE	AEROGENES TYPE WITH SHEEN	DOUBTFUL AEROGENES TYPE	AEROGENES POSITIVE TYPE	TYPICAL AEROGENES TYPE	PIN POINT METALLIC SHEEN TYPE	TOTAL ALL TYPES
Number of colonies fished...	181	33	169	29	125	46	95	8	686
Number of colonies producing indol	155	22	98	16	37	15	29	1	373
Per cent of colonies producing indol................	85 7	66 7	58	55.2	29 6	32 6	30.5	12 5	53 4

ing to Standard Methods (1923) on the basis of the methyl-red
test, Voges-Proskauer reaction, adonitol fermentation and gelatin
liquefaction. Classified by these criteria their cultures gave the
following indol reactions:

	CLASSIFICATION			
	Bact. coli	*Bact. aerogenes*	*Bact. cloacae*	Atypical
Number of cultures................	695	321	20	180
Per cent indol +....................	87	23.4	30	Not given

These cultures were fished from colonies on Endo plates and
were not purified before they were differentiated. The percent-
age indol production found in their *Bact. coli* and *Bact. aerogenes*
sections are remarkably close to that found by us for typical
Bact. coli and typical *Bact. aerogenes* colonies on E.M.B. agar
as shown in table 4. Since we know that our colonies were not
pure we believe that the variability of the indol reactions in
Bahlman and Sohn's cultures may also be explained in part by
mixed cultures.

We have shown that the indol test is extremely sensitive and

that the production of indol by indol formers is not easily inter-
fered with, even though they are in the minority, by non indol-
producers.   We have also found that the ability to produce indol
is not easily acquired or lost by a pure strain.   These facts sup-
port the view that variability in indol production in the *Bact.
aerogenes* section may often be due to *Bact. coli* contamination.
It is well known that *Bact. cloacae* produces indol.   Bahlman and
Sohn's data indicate that the incidence of isolation of these strains
is low in routine work.   We have also found that indol is some-
times formed in *Bact. aerogenes* cultures due to contamination
by spore forming organisms.   When such cultures are purified,
the *Bact. aerogenes* show their normal differential reactions.

In concluding we reaffirm our conviction of the value and
importance of the indol test as held by Houston, Hicks, Ragha-
vachari and others.   The test requires the shortest incubation
period of any of those described.   It is simple, positive and very
sensitive.   The indol once produced is stable in the alkaline
tryptophane medium and the reaction is not easily interfered
with.   Mixtures may therefore be easily indicated and detected
with it.   The ability to produce indol is not easily acquired or
lost.   Danger of pseudo negatives with fecal *Bact. coli* strains
has been overemphasized.   Though some strains of *Bact. aero-
genes* sections may produce indol their incidence is low.   This
test with the other three described may be used in conjunction
with colony appearance and the Gram stain report to differentiate
the group and to detect mixed cultures which would otherwise be
classed as pure.

CONSTANCY OF BIOCHEMICAL REACTIONS

Before discussing the interpretation of results with the four
tests described we wish again to stress the fact that these reactions
are all very constant in pure cultures.   The coli-aerogenes group
may form dissociants on agar plates which would not be recog-
nized as belonging to the parent strain.   We have found that
these dissociants, however, produce the same reaction combina-
tion of the aforesaid biochemical tests as the parent strain.
Many of our water samples may contain such dissociants and it

seems likely that this sometimes hinders the proper macroscopic differentiation of certain colony types on isolation plates. To show the variation in colony production by *Bact. coli*, the types of colonies produced by the 38 pure strains that were used in some of our experiments are shown below:

B. Coli strains
Lactose+, Indol+, M.R.+, V.P.−, Cit.−

TYPE 1— TYPICAL B. COLI COLONIES ONLY	TYPE 2— TYPICAL B. COLI AND BLUE COLONIES	TYPE 3— BLUE TYPE COLONIES ONLY	TYPE 4— TYPICAL COLI AND DOUBTFUL AERO- GENES TYPE COLONIES	TYPE 5— AERO- GENES SHEEN TYPE COLONIES	TYPE 6— TYPICAL AERO- GENES AND BLUE COLONIES	TYPE 7— DOUBTFUL AERO- GENES TYPE COLONIES	TYPE 8— BLUE AND DOUBTFUL AERO- GENES TYPE COLONIES
10	6	4	11	2	2	1	2

It is certain that strains in types 5, 6, 7 and 8 would not be recognized as *Bact. coli* and a single differential test with the colonial picture would be confusing. When the four differential tests above always give the *Bact. coli* reaction combination, there can be little doubt of the biochemical classification regardless of colonial appearance. We have been able to alter colonial characteristics of pure strains by various treatments but in no case have we been able to alter the biochemical reaction combination obtained with the above described tests. We have found this true for both the *Bact. coli* and *Bact. aerogenes* sections. We have therefore the utmost confidence in the reliability and constancy of these differential tests.

DIFFERENTIATION

During 1928 and 1929, 1488 cultures isolated from water were differentiated using the M.R., V.P. and Koser's citrate tests. Of these, 150 were isolated from the same number of samples from a deep drilled well at Riverside, Illinois. The remainder came from Lake Michigan water in all stages of treatment from raw to chlorinated tap and from points on the lake from Waukegan, Illinois to Gary, Indiana. The majority of these cultures were tested after they had been completely confirmed by standard methods. In some cases the cultures were picked direct from

isolation plates following positive presumptive tests. Simply stating the percentage of positive or negative reactions obtained with each of the tests may be very misleading. We therefore prefer to give the actual combination of reactions obtained with each culture. The results are shown below, classified according to the eight different possible combinations of reactions with these three tests. In this table the order of the reactions given is always from left to right for the methyl-red test, the Voges-Proskauer reaction and Koser's citrate test respectively.

	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6	GROUP 7	GROUP 8
Reaction combinations.....	+ − −	+ + +	+ − +	+ + −	− − +	− + +	− + −	− − −
Number of cultures........	606	66	399	22	153	92	22	128
Possible interpretation.....	*Bact. coli*	These reaction combinations may be interpreted in several different ways				*Bact. aerogenes*	Not in coli-aerogenes group	Not in coli-aerogenes group

These results were rather disappointing. The numbers in group 7 and 8 were surprisingly large. Eliminating these from consideration there remain 1338 cultures which may be considered to belong to the coli-aerogenes group. Of these, 606 or 45 per cent were apparently *Bact. coli* while only 92 or 7 per cent gave the correct tests for *Bact. aerogenes*. No definite interpretation could be made of the remaining cultures. Groups 2 and 3, representing about 35 per cent of the total, may be interpreted as *Bact. coli*, *Bact. aerogenes*, a mixture of both, or as either one with a non-member of the group as a contaminant. These data show that even though three tests were made on routine confirmed *coli*, *aerogenes* cultures a large proportion of the cultures could not be definitely classified.

During 1930 the studies on the indol, methyl-red, Voges-Proskauer and Koser's citrate tests, that have already been described, were made and these tests were applied to 2,093

cultures isolated from water. There are sixteen possible combinations of the above four differential tests as follows:

	REACTION COMBINATION NUMBER															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Indol	−	+	+	+	+	+	−	−	−	−	+	+	+	−	−	−
Methyl red	+	+	+	+	−	−	+	+	−	−	+	−	+	−	−	−
Voges-Proskauer	−	−	−	+	+	−	−	+	+	−	−	+	+	+	+	−
Koser's citrate	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−

Less than 2 per cent of all the cultures were found in the last six classifications shown above. These strains were distributed as follows:

	REACTION COMBINATION NUMBER					
	11	12	13	14	15	16
Reaction combination in following order: Indol, M.R., V.P., citrate	+ − − −	+ + + −	+ − + −	− + + −	− − + −	− − − −
Number of cultures of the 2,093	7	3	5	3	7	15
Per cent of total cultures	0.33	0.14	0.24	0.14	0.33	0 72

In this table and hereafter in the paper these biochemical differential reactions will be given in the following order Indol, Methyl-red, Voges-Proskauer and Koser's citrate reaction. For instance − + − + means that the culture was indol negative, methyl red positive, Voges-Proskauer negative and citrate positive.

While the cultures shown in this table were not studied intensively we believe that reaction combinations eleven to fourteen, inclusive, may be caused by mixed strains and that occasionally *Bact. coli* may be present in any of them. Usually, these combinations indicate the absence of all members of the coli-aerogenes group. The last two reaction combinations indicate strains that do not belong to the coli-aerogenes group. Since cultures having these reactions were comparatively rare we shall not consider them further.

The remaining 2,053 cultures as separated according to their

method of isolation and their differential reactions are shown in table 5.   The first group of cultures designated −P−included only cultures which were confirmed by the routine procedure of standard methods and had been reported Gram-negative, pure and non-spore-forming.   The second group designated + or −M− or sp. included cultures which were completely confirmed by the Standard Methods but were reported as mixtures when Gram-stained smears were examined microscopically.   These mixtures frequently contained spores and in some cases Gram-positive organisms.   Pure Gram-positive cultures or cultures

TABLE 5

Differential reactions of confirmed coli-aerogenes group cultures isolated from water

DESCRIPTION OF ISOLATED CULTURES	TOTAL CULTURES	REACTION COMBINATIONS									
		1	2	3	4	5	6	7	8	9	10
		−+−−	++−−	++−+	++++	+−++	+−−+	−+−+	−+++	−−++	−−−+
−P− ..	850	15	334	113	63	67	25	71	20	104	38
+ or −M− or sp ...	368	18	98	45	26	11	17	68	20	49	16
Coli-aerogenes type, E.M.B ...	541	17	213	30	15	22	12	49	20	127	36
Coli-aerogenes type, cyanide citrate....	294	9	78	17	10	8	8	35	6	99	29
Total.....	2053	59	723	205	114	108	57	223	66	379	119

containing only spore formers were naturally not included in this group.   We have studied this group of 368 cultures to obtain a more reliable index of what such cultures might represent.   This number of Gram-positive or negative mixed spore or non-spore forming cultures was accumulated from about 2,500 completely confirmed cultures.   The 541 cultures from E.M.B. agar plate colonies were very carefully picked from selected E.M.B. plates.   These cultures do not represent a true picture of all cultures obtained and confirmed as coli-aerogenes for plates containing non-characteristic colonies were not included in this group.   The 294 cultures picked from Noble's ferrocyanide citrate pour plates

were obtained from a smaller number of sampling points and from raw water only. These four groups of cultures should therefore not be compared as representing the same group of water samples. The results by these methods of isolation do show that all of the various combinations of differential reactions are obtained in each case. The percentages of the various differential combinations obtained on these cultures are shown in table 6. It will be noticed in this table that the percentages of the most common *Bact. coli* as indicated by the indol +, M.R. +, V.P. − and citrate − reaction combination and also *Bact. aerogenes* or *Bact. cloacae* as

TABLE 6

Percentage of various differential reaction combinations obtained with coli-aerogenes cultures isolated from water

REACTION COMBINATIONS — Indol / M.R. / V.P. / Citrate	−P−	−M−, −Msp.	E.M.B. COLONIES	CYANIDE CITRATE COLONIES	ALL CULTURES
− + − −	1.7	4.9	3.1	3.1	2.9
+ + − −	39.3	26.7	39.4	26.5	35.2
+ + − +	13.3	12 2	5.5	5.8	10 0
+ + + +	7.4	7.1	2.8	3 4	5.5
+ − + +	7.9	3.0	4.1	2.6	5.3
+ − − +	2.9	4.6	2.2	1.0	2.8
− + − +	8.4	18.5	9.0	11.9	10.9
− + + +	2.3	5.4	3.7	2.0	3.2
− − + +	12.3	13.3	23.5	33.7	18.5
− − − +	4.5	4.3	6.7	9 8	5.8

indicated by the indol −, M.R. −, V.P. + and citrate + reaction combination are surprisingly low. It may be pointed out that the percentage of these *Bact. coli* isolated by the routine method from all classes of water was higher than the percentage of these organisms isolated from the ferrocyanide citrate pour plate medium from raw waters only. This is easily understood when it is recalled that the standard method recommends that the most typical colonies be picked. Consequently the *Bact. coli* types are usually picked on routine plates and the *Bact. aerogenes* types are neglected unless the plates only contain such types. In the case of direct planting of samples on ferrocyanide citrate

plates both types are more often found on one of the dilutions of every sample and in all such cases both types were fished.

The total percentage of the common *Bact. coli* and *Bact. aerogenes* types as indicated by the reaction combinations $+ + - -$ and $- - + +$ seem to be very low. We have therefore assembled the data from several observers on this point in table 7. This table indicates that Lewis and Pittman (1928) obtained a variation from 39 per cent in water of high sanitary quality to 76

TABLE 7

Percentage of Bact. coli and Bact. aerogenes cultures obtained by biochemical differentiation by various observers

OBSERVER	SOURCE AND TYPE OF WATER	TOTAL COLI-AERO-GENES STRAINS ISOLATED	PERCENTAGE THAT DIFFER-ENTIATED AS REGULAR BACT. COLI AND BACT. AEROGENES
Bahlman and Sohn............	Ohio River, raw, settled, filtered and chlorinated	1,223	71.4
Lewis and Pittman..........	Polluted water	83	76.0
	High sanitary quality raw	196	39.0
Sanitary district of Chicago..	Lake Michigan, raw, cyanide citrate cultures	294	60.5
	Selected E.M.B. plate cultures	574	63.0
	Lake Michigan, raw, settled, filtered, and chlorinated, $-P-$	850	51.5
	Same as above but $+$ or $-$ mixed $-$ or sp.	368	40.0

per cent in polluted water of these common regularly reacting *Bact. coli* and *Bact. aerogenes* strains. Even in water samples originating from the highly polluted Ohio River, Bahlman and Sohn obtain only 71.4 per cent of *Bact. coli* and *Bact. aerogenes* which conformed to these strict definitions. The percentages of such strains that were obtained from Lake Michigan are within the variations obtained by Lewis and Pittman.

Referring to table 5 we will assume for the time that reaction

combinations Nos. 1 and 2 are *Bact. coli* and that 9 and 10 are *Bact. aerogenes.* How are we going to interpret the intermediate reaction combinations of numbers 3 to 8 inclusive? The total percentage of these intermediates found in our groups of cultures were as follows:

GROUP	−P−	+ OR −M− SP.	E.M.B. COLONIES	FERRO-CYANIDE CITRATE COLONIES
Percentage intermediates............	42 2	50.8	27.3	26.7

This shows that the lowest percentage of these intermediates was found in Noble's ferrocyanide citrate pour plate cultures and the highest in cultures isolated by the standard method but reported mixed after microscopic examination. From this we infer that these intermediate reaction combinations may contain mixtures of strains.

PURIFICATION OF INTERMEDIATE REACTION COMBINATION CULTURES

To study the apparent intermediate strains, 266 such cultures were purified. This group included 28 cultures that gave the − − − + reaction combination. About fifty cultures that were purified originated in the E.M.B. agar colony culture group. Fifty more were cultures that were completely confirmed but were reported as mixed when examined microscopically. The remainder and largest portion were completely confirmed and reported as Gram-negative, pure, non-spore-forming. While it might be desirable to consider the results of purification of each of these sections separately we have summarized them in one group. This was done because the results from all sections were very much alike and also to reduce the number of tables and simplify the presentation.

Figure 3 shows graphically the methods of purification used in the work. The diagrams are almost self-explanatory and need little comment. As many different colonies as were present were picked from the first reisolation plate so that four or five subcultures were usually carried through one of these methods for each

of the original 266 cultures.   We believe that the first method is the least satisfactory.   Some *Bact. coli* strains were lost by the second method where they were carried through all the media at 46°C.   Experience with this method also showed that some *Bact. aerogenes* grew well at 46°C., even on E.M.B. agar plates and in brilliant green broth.   The experiments with method two incidentally indicated that Leiter's (1929) report, that implantation of water portions into glucose broth and incubation at 46°C. resulted in an index indicative of only the fecal *Bact. coli* strain

TABLE 8

Result reaction combination after purification of cultures having intermediate differential reaction combinations when isolated

ORIGINAL DIFFER-ENTIAL REACTION COMBINA-TIONS	ORIGINAL NUMBER OF CULTURES	BIOCHEMICAL REACTIONS OF STRAINS AFTER PURIFICATION									
		-+--	++--	++++	++-+	+-++	+--+	-+-+	-+++	--++	---+*
++++	47	2	14	8	8	4	1	6	6	17	1
++-+	71	1	24	13	15	5	1	14	6	12	6
+-++	50	1	18	1	3	4	0	5	1	32	2
+--+	28	0	11	2	0	5	0	1	0	15	1
-+-+	31							24		6	1
---+	28							2	1	14	13
-+++	11							2	5	5	
Total	266	4	67	24	26	18	2	54	19	101	24

* Strains having this reaction combination after purification usually do not ferment lactose and do not belong to the coli-aerogenes group.

from warm-blooded animals, is not correct.   Method four, utilizing direct planting of the culture to be purified into Noble's ferrocyanide citrate agar has the advantage of requiring the shortest time to obtain the final results.   It requires more skill and manipulation time, however, and has other disadvantages. We prefer and recommend method three as being very simple, easy to manipulate, and capable of excellent results and we have adopted it for routine purification studies.

    For simplicity the results of the purification have been summarized in tables 8, 9 and 10.   Table 8 shows the reactions of the

TABLE 9

Summary of results after purification of cultures having intermediate differential reactions

ORIGINAL DIFFERENTIAL REACTION COMBINATIONS					TOTAL NUMBER CULTURES PURIFIED	BIOCHEMICALLY DIFFERENT STRAINS ISOLATED AND DIFFERENTIATED AFTER PURIFICATION		CULTURES FROM WHICH TWO OR MORE WERE ISOLATED		CULTURES HAVING NO CHANGE IN REACTIONS AFTER PURIFICATION		NUMBER OF STRAINS ISOLATED, NOT BELONGING TO COLI-AEROGENES GROUP	
Indol	M.R.	V.P.	Citrate			Number	Percentage*	Number	Percentage*	Number	Percentage*	Number	Percentage†
+	+	+	+		47	67	143	19	40	7	15	1	1.5
+	+	−	+		71	97	137	24	34	11	15	6	6.2
+	−	+	+		50	67	134	15	30	3	6	2	3.0
+	−	−	+		28	35	125	7	25	0	0	1	3.6
−	+	−	+		31	31	100	0	0	24	77	1	3.2
−	−	−	+		28	30	107	2	7.1	11	39	13	43.0
−	+	+	+		11	12	109	1	9	4	36	0	0
Total..					266	339	127	68	26	60	23	24	7.1

* Percentage of original cultures.
† Percentage of total strains after purification.

TABLE 10

Classification of intermediate reacting cultures before and after purification

REACTION COMBINATIONS				BEFORE PURIFICATION		AFTER PURIFICATION	
Indol	M.R.	V.P.	Citrate	Number	Percentage	Number	Percentage
−	+	−	−	0	0	4	1.2
+	+	−	−	0	0	67	19.8
−	−	+	+	0	0	101	29.8
+	+	+	+	47	17.7	24	7.1
+	+	−	+	71	26.6	26	7.6
+	−	+	+	50	18.8	18	5.3
+	−	−	+	28	10.5	2	0.6
−	+	−	+	31	11.7	54	15.9
−	+	+	+	11	4.2	19	5.6
−	−	−	+	28	10.5	24·	7.1
Total........				266	100	339	100

various strains that were obtained after purification. It fails to show how many cultures were not changed by the purification method. It represents the total resultant picture, however, showing that many of these originally irregular or intermediate reacting cultures conformed to the more common *Bact. coli* and *Bact. aerogenes* reacting strains after purification.

Table 9 shows the increase in the total number of different strains obtained as a result of purification. It indicates that from cultures which gave both positive indol and citrate reactions, two strains were isolated from 25 to 40 per cent of the time depending upon the reaction combination. It shows that except for the $- + - +$ reaction combination the percentage of these cultures which had no change in their differential reactions as a result of purification was low. Table 10 shows the classification and the percentages of the total group of cultures before and after purification. This indicates that, after purification, 50.8 per cent total strains obtained are classified as *Bact. coli* and *Bact. aerogenes* having the characteristic reaction combinations.

DISCUSSION OF RESULTS OF PURIFICATION STUDY

Our data indicate that cultures having the reaction combination $- - - +$ usually contain *Bact. aerogenes*, a non-member of the group or both. Such cultures must be purified and examined again for proper classification. Occasionally these cultures may contain the $- + - +$ reacting strain.

While comparatively few $- + + +$ reacting cultures were studied, three strains were most commonly obtained from them. About half of these cultures showed no change in their reactions by the ordinary purification processes. Pure *Bact. aerogenes* were obtained in four cases and in one case two strains were separated, one of which was *Bact. aerogenes* and the other the $- + - +$ intermediate strain. We also believe that some of these $- + + +$ reactions are caused by mixtures of *Bact. aerogenes* and glucose-fermenting organisms which do not belong to the coli-aerogenes group. This reaction combination may also result from *Bact. aerogenes* and *Bact. coli* strains which do not produce indol, or it may result from *Bact. aerogenes* and *Bact. coli* that are weak

indol producers and are outgrown by the *Bact. aerogenes* in the tryptophane broth as was shown in the indol section of this paper. We believe that this combination invariably represents a mixture of strains.

Those cultures having $- + - +$ reaction combinations were found to be apparently pure strains in over 70 per cent of the cases. In six cases regular *Bact. aerogenes* reacting strains were obtained after purification. This change may be considered as due to the elimination of M.R. $+$ contaminants from originally mixed cultures or it may be due to the cause suggested by Koser (1924) when he observed similar changes in organisms isolated from soil. Koser considered such changes in reaction to be the result of slow growing *Bact. aerogenes* strains in which the secondary or alkaline fermentation was speeded as a result of laboratory cultivation. We believe that either of these explanations is tenable in some cases. We conclude, therefore, that while some $- + - +$ cultures contain *Bact. aerogenes* ($- - + +$) the majority of them remain unchanged. Such strains have been reported isolated from soil by Koser (1924) (1926) and from water by Bardsley (1926) Raghavachari (1926) and Lewis and Pittman (1928). We have found these strains extremely rare in human and animal feces (see table 12). Recently Werkman and Gillen (1931) reported that such Coli-aerogenes intermediates fermented glycerol with the production of trimethylene glycol. Typical *Bact. coli* and *Bact. aerogenes* do not have this property and they, therefore, propose generic recognition of this group under the name Citrobacter.

INTERMEDIATES THAT PRODUCE INDOL AND UTILIZE CITRATE

Purification greatly reduced the number of cultures that produced indol and utilized citrate as a sole source of carbon. Every culture which had the reaction combination $+ - - +$ was changed by the purification process. We believe that this reaction combination is always indicative of a mixed culture. Table 8 shows that *Bact. coli* was isolated almost as frequently as *Bact. aerogenes*. A culture having $+ - + +$ reactions was the third most frequent result of purification of the above group.

Cultures having the reaction combination + − + + are ordinarily interpreted as being *Bact. aerogenes* or *Bact. cloacae* which produce indol. When 50 such cultures were purified, eleven of them were found to contain both *Bact. coli* and *Bact. aerogenes*. As table 8 shows, fifty of these cultures yielded 32 *Bact. aerogenes* strains and 18 *Bact. coli* strains. One such culture was also found to contain *Bact. coli* and the − + − + strain described above. Only four cultures or 6 per cent of the strains obtained from this group remained with the same reaction after purification. Another 6 per cent of these cultures were altered in some way by purification so that they produced the other intermediate reactions still to be discussed. We conclude, therefore, that *Bact. aerogenes* or *Bact. cloacae* that produce indol are very rarely encountered in the surface waters of the Chicago area.

The cultures having the reaction combination + + − + may be generally considered as *Bact. coli* which utilize citrate. The − + − + group becomes indistinguishable from this group if the indol test is omitted. Table 5 shows that this was one of the most common intermediate reacting groups found. Table 8 shows that when such cultures were purified strains of *Bact. coli*, *Bact. aerogenes* or the − + − + strains were frequently obtained. About 15 per cent of the strains obtained after purification of this group had the same reactions as at the start and another 20 per cent other intermediate reactions. Our results seem to indicate that pure strains having this reaction may be occasionally encountered in surface waters. We believe that such strains are of soil rather than of fecal origin.

The only intermediate group cultures still to be discussed are those giving positive reactions for all of these tests. Table 5 shows a surprising number of cultures having these reactions when isolated. Such cultures are seldom reported in the literature, probably because all of these tests are not usually made on routine cultures from water. Apparently Lewis and Pittman (1928) did not encounter such cultures in Texas, but Koser (1926) did report an atypical strain isolated from soil which may have been identical with these. Purification of our cultures resulted in the separation of a large percentage of *Bact. coli, Bact. aerogenes*

and the intermediate $- + - +$ strains. Some of the cultures having all positive differential tests were not affected by the purification process and some strains, with other intermediate reactions, were obtained.

Our study of intermediate coli-aerogenes group cultures that produce indol and utilize citrate leads us to conclude that such cultures, when isolated by the present standard method from surface water samples, are usually mixtures of two or more strains. From 65 such cultures, two strains and in three cases, three strains were separated. Twenty-five different combinations of strains were obtained. The most frequent combination of strains was the common *Bact. coli* and *Bact. aerogenes*. This combination was separated from 29 of these cultures. In one cases, *Bact. coli*, *Bact. aerogenes* and the $- + - +$ intermediate strain were separated from one colony.

APPLICATION OF RESULTS OF PURIFICATION EXPERIMENT ON THE ORIGINAL INTERMEDIATE CULTURES

It is unfortunate that each culture listed in the intermediate groups shown in table 5 was not purified. Due to the amount of work involved this could not be done. We are assuming that approximately the same results would have been obtained with the entire group as with the 266 cultures that were purified. Each group of intermediates in table 5 was redistributed in the same way as the same groups in table 8. Since we have concluded that the $+ - - +$ reaction combination never represents a pure strain, this reaction combination was not included after adjustment for purification. The total results before and after adjustment for purification are shown in table 11. This table shows that purification resulted in an increase of 174 *Bact. coli* strains and an increase of 308 regular *Bact. aerogenes* strains, indicating that about 18 per cent of the total *Bact. coli* strains and about 45 per cent of the total regular *Bact. aerogenes* strains isolated were in mixtures when first purified.

It will be noticed that the percentage of $+ + - +$ intermediate cultures, the $+ + + +$ atypical cultures and the $+ - + +$ (*Bact. aerogenes*, producing indol) cultures have been reduced con-

siderably. The $+ + - +$ strains resemble *Bact. coli* in most of their characteristics, but it is difficult to determine whether they should be classified with *Bact. coli* or with the intermediate strains.

TABLE 11

Differential reactions of the coli-aerogenes cultures from water before and after purification

	REACTION COMBI-NATIONS				NUMBER OF CULTURES		PERCENTAGE OF CULTURES		SUGGESTED INTERPRETATION
	Indol	M.R.	V.P.	Citrate	Original	After purification	Original	After purification	
1	−	+	−	−	59	69	2.9	3.2	Coli
2	+	+	−	−	723	887	35.2	41.3	Coli
1 and 2..... ...					782	956	38.1	44.5	Total coli
3	+	+	−	+	205	68	10 0	3.2	Intermediate
4	−	+	−	+	223	261	10 9	12.1	Intermediate
3 and 4					428	329	20.9	15 3	Total intermediate
5	+	−	−	+	57	0	2 8	0	Atypical
6	+	+	+	+	114	62	5 5	2.9	Atypical
7	+	−	+	+	108	43	5.3	2 0	Aerogenes-cloacae producing indol
8	−	+	+	+	66	68	3.2	3.2	Aerogenes-cloacae possibly contaminated
9	−	−	+	+	379	687	18.5	32.1	Aerogenes-cloacae
7, 8 and 9......					553	798	27.0	37.3	Total Aerogenes cloacae
10	−	−	−	+	119	87*	5.8	—	Possibly Aerogenes or cloacae
Total.........					2,053	2,145			Coli-aerogenes group

* These cultures were not *Bact. aerogenes* or *Bact. cloacae* and are therefore not included in the Coli-aerogenes group total after purification.

A study of the coli-aerogenes strains present in feces should assist in the interpretation of these results.

COLI-AEROGENES GROUP IN FECES

While numerous investigators have reported on the abundance of *Bact. coli* and the low percentage of *Bact. aerogenes* present in

feces, they have not used our four tests in differentiating the two strains. It is well known that *Bact. coli* is the predominating organism of feces. Our study of growth rates in lactose broth (see first section of this paper) indicates that under this condition *Bact. aerogenes* will very rarely be isolated by the standard method. This point is very well illustrated by the recent work of Brown and Skinner (1930) who reported that, "only *B. coli* (never *B. aerogenes*) were found in human feces." However, if the fecal suspensions are planted directly into Noble's ferrocyanide citrate agar and a number of every type of colony on the more crowded plates is carefully picked, the coli-aerogenes organisms less frequently found in feces will occasionally be isolated. Another and simpler method that we have used to isolate these minority fecal strains depends upon the more rapid growth of such strains in Koser's citrate solution. Dilutions of the fecal suspensions similar to those ordinarily planted in lactose broth are also planted in Koser's citrate solution. After twenty-four hours incubation at 37°C. the highest dilution with visible growth in the citrate solution and the next highest dilution are streaked on E.M.B. agar plates. Representative colonies of all types are then fished for study. Figure 1 shows E.M.B. plates that were obtained by this method and the standard method from

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FIG. 1. CULTURES ISOLATED FROM HUMAN FECES. DIFFERENTIAL
REACTIONS OF COLONIES

Plates 1, 2, and 3 streaked from citrate cultures

Plate 1				Plate 2				Plate 3		
Colony number	Type	Reaction		Colony number	Type	Reaction		Colony number	Type	Reaction
1	TC	+−++		1	BC	++−−		1	TC	++−−
2	AS	+−++		2	TC	++−−		2	TA	+−−+
3	A+	−−++		3	A±	−+−+		3	A±	++−−
4	A±	−+−+		4	A+	−+−+				
5	A+	−−++		5	A+	−+−+				
6	A±	−+−+								

Plates 4, 5, and 6 streaked from lactose broth cultures

Plate 4				Plate 5				Plate 6		
Colony number	Type	Reaction		Colony number	Type	Reaction		Colony number	Type	Reaction
1	AS	−+−−		1	TC	++−−		1	AS	++−−
2	A±	++−−		2	TC	++−−		2	AS	++−−
3	A±	++−−		3	BC	++−−		3	AS	++−−
4	BC	++−−								
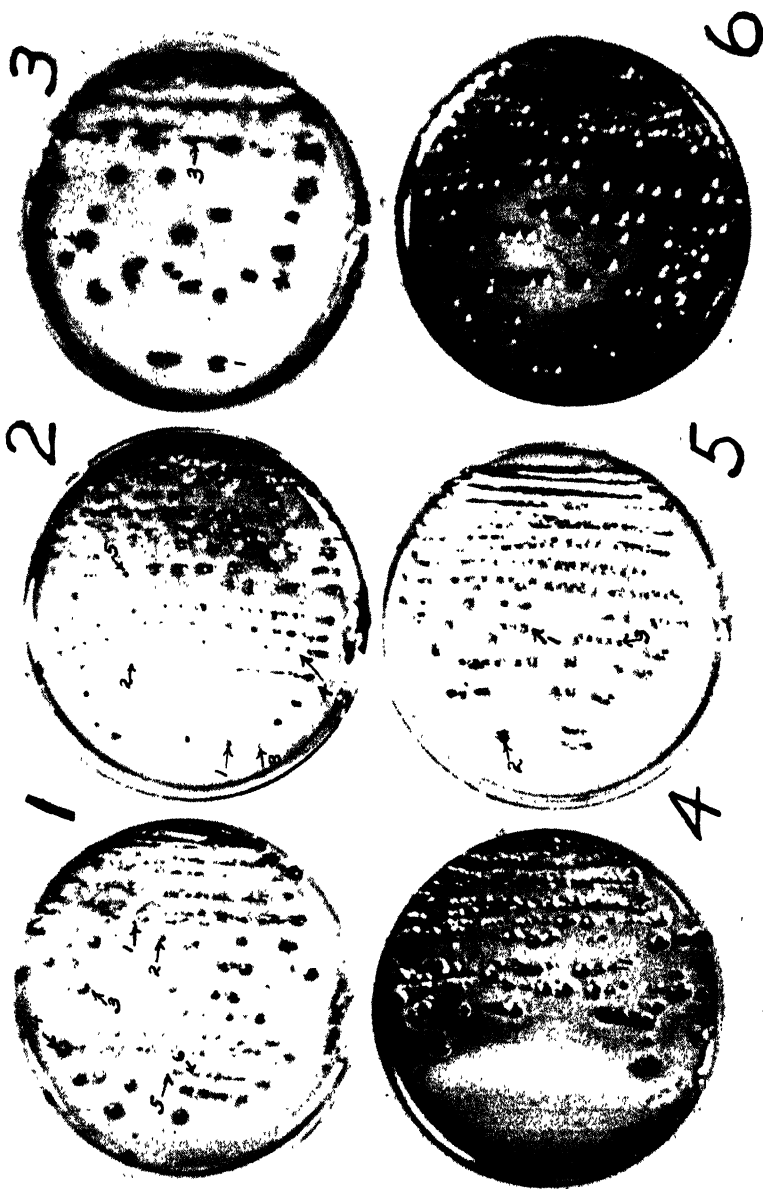
FIG. 1

feces with the colonies that were picked and the differential results obtained. Using this method and Noble's ferrocyanide citrate direct pour plates, we studied 32 samples of feces from eleven persons and ten animals. The animal feces included samples from one guinea pig, two rabbits, three dogs, two sheep and two monkeys. The results obtained on 486 colonies isolated from these samples are shown in table 12. The data from this careful study of a limited number of fecal samples show that apparently all varieties of strains were obtained from the isolated colonies. After purification of all intermediates, all strains were classified

TABLE 12

Differential reactions of the coli-aerogenes cultures from human and animal feces

REACTION COMBINATIONS				NUMBER OF CULTURES		PERCENTAGE OF CULTURES	
Indol	M.R.	V.P.	Citrate	Original	After purification	Original	After purification
−	+	−	−	56	56	11 5	11.5
+	+	−	−	402	406	82 9	83 2
+	+	−	+	3	0	0 6	0
−	+	−	+	9	10	1.8	2 0
+	−	−	+	2	0	0 4	0
+	+	+	+	2	0	0 4	0
+	−	+	+	2	0	0 4	0
−	+	+	+	0	0	0	0
−	−	+	+	8	16	1.6	3 3
−	−	−	+	2	0	0 4	0
Total.......				486	488	100	100

as *Bact. coli*, *Bact. aerogenes* or the − + − + intermediate strain. The last two types were very much in the minority. The percentages of the types given in the tables are not at all indicative of the ratios of these organisms present in feces. They simply apply to this group of cultures as isolated by the methods described. The study indicates that *Bact. aerogenes* and the − + − + intermediates are very rare and that other atypical strains must be even more rare in feces. Since we were not able to find a single + + − + strain in feces we tentatively conclude that such strains isolated from water probably originate from

other sources and therefore should be classified with the $- + - +$ intermediates rather than with *Bact. coli.* It should be pointed out that all of the non-indol-producing *Bact. coli* in this experiment were obtained from one individual. The samples from this person contained no *Bact. aerogenes* or intermediates but contained both types of *Bact. coli* with the non-indol-producing variety making up the great majority of the population. On E.M.B. plates these non-indol-producing *Bact. coli* yielded large aerogenes sheen type colonies and on Noble's ferrocyanide citrate plates they also produced very large colonies resembling *Bact. aerogenes.* The inclusion of three samples of feces from this individual, who apparently is not representative in this respect, probably accounts for the rather high percentage (11.5 per cent) of these strains isolated.

SUMMARY

Preliminary enrichment in lactose broth

1. Our study of preliminary enrichment has indicated that when almost equal numbers of *Bact. coli* and *Bact. aerogenes* are present in water, which is often the case, there is a general tendency for one organism to outgrow the other. It is impossible to predict which strain will gain the ascendancy. The ascendancy of one of the strains depends upon three factors, first, the initial physiological condition of the strains (for this determines the relative length of the lag phase), second, the relative growth rates of the strains in their logarithmic growth phase, third, the relative sensitivity of the strains involved to the products of metabolism. The ascendancy of one of the strains takes place during the first twenty-four hours incubation at 37°C. and the relative numbers of the organisms remain about the same during the second twenty-four hours. The tendency of one organism to overgrow the other is reflected in the E.M.B. agar plate results by Standard Methods on routine samples.

Isolation of coli-aerogenes cultures

1. Levine's eosine-methylene-blue-agar or Skinner and Murray's modification of it appeared to be the best medium for isolation

of the group. Neither of these media is ideal, however. The results obtained with Levines E.M.B. agar are also applicable to other streaked isolation media.

2. Single streaked isolation plates sometimes indicate pure cultures when the cultures are actually mixed. Contaminants of coli-areogenes cultures sometimes mask the colonial characteristics of the coli-aerogenes strains. Many isolated colonies on streaked plates that are considered pure are in reality contaminated with other organisms.

3. Due to the incidence of mixed colonies, the masking effect of contaminants, the variety of colonies produced by pure *Bact. coli*, pure *Bact. aerogenes* and possible dissociants, macroscopic differentiation of routine E.M.B. agar colonies is not successful. It is absolutely impossible to differentiate macroscopically, colonies of the socalled intermediate group strains. Many such strains produce colonies which are similar to typical *Bact. coli* while others are apparently identical with *Bact. aerogenes* in colony appearance (see fig. 2).

4. Microscopic examination of Gram-stained smears does not reveal all coli-aerogenes group cultures that are mixed with other Gram negative non-spore-forming organisms. It never reveals mixtures of *Bact. coli* and *Bact. aerogenes* or mixtures of either of these strans with the intermediate soil forms.

Biochemical differential tests

1. The indol, methyl-red, Voges-Proskauer and Koser's citrate biochemical differential tests were studied with coli-aerogenes strains and with *Bact. coli* and *Bact. aerogenes* mixtures. This study indicated that the methyl-red and Voges-Proskauer tests were the least reliable and were more likely to give misleading or incorrect results due to contaminants. The indol and the citrate tests, on the other hand, are very reliable. Positive tests are practically always obtained when organisms giving these tests are present even though they are initially greatly in the minority. This is not true for the M.R. and V.P. tests. The indol and citrate tests can therefore be used to indicate the possible presence of contaminants. The data indicate that the use of the M.R.
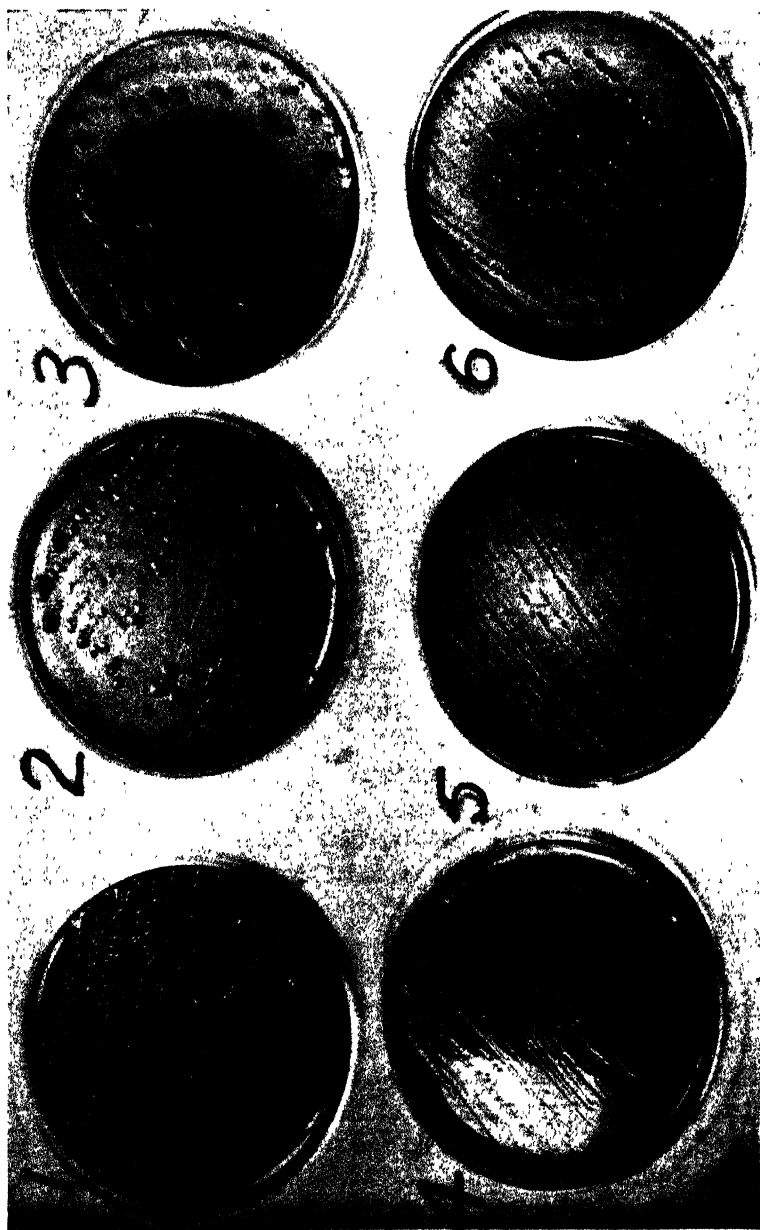
FIG. 2. SHOWING PURE STRAIN COLONIES ON E.M.B. AGAR PLATES

All of these cultures have the following differential reactions: Indol −, M.R. +, V.P. −, citrate +. Plates 1, 2, and 3 have aerogenes colony characteristics. Plates 4, 5, and 6 have coli colony characteristics.

and V.P. tests on routine samples is not satisfactory. Even with mixtures, perfect correlation of these tests will usually be obtained and while the result will usually indicate the predominating organism it will give no indication that the culture is actually a mixture. With these tests intermediate soil strains will be grouped with the fecal *Bact. coli* rather than with the soil forms where they belong. If only two tests are to be used the indol and citrate tests are preferable. We consider the V.P. test the least valuable of the four for routine work where many of the cultures are usually mixtures. The four tests used together are superior to any three and make possible a more accurate and satisfactory interpretation.

2. The reaction combinations obtained with the above four tests on pure *Bact. coli* and pure *Bact. aerogenes* strains are very constant and were not altered by laboratory treatment. The reaction combinations of *Bact. coli*, intermediate and *Bact. aerogenes* strains are different with these tests and they remained different regardless of age or treatment. These tests, therefore, serve as a superior criterion of differentiation.

Differential test interpretation

1. There are ten combinations of the above differential reactions that are commonly encountered in the examination of a large number of surface water cultures. These combinations are listed on page 177 with their interpretations and the most common habitat of the individual strains they represent.

All those combinations which are sometimes mixtures must be purified and differentiated again before the correct interpretation can be made.

2. Several methods of purification of cultures having irregular combinations that indicate possible mixtures were studied. The method of transfer to lactose broth followed by reisolation is not satisfactory. The following method with all subcultures incubated at 37°C. is recommended. Culture in tryptophane broth two to three hours and streak on E.M.B. Incubate plates for twenty to twenty-four hours. Repeat this process three times, each time fishing subcultures from all types of colonies on the

E.M.B. plates. Then fish all subcultures to differential media, lactose broth and agar slants. This method will not purify and yield coli-aerogenes strains from all mixtures in which the coli-aerogenes organisms are present in the minority. In some such instances direct planting in Noble's ferrocyanide citrate agar produces successful coli-aerogenes isolations.

| REACTION COMBINATIONS | | | | INTERPRETATION WHEN ISOLATED FROM WATER BY THE STANDARD METHOD | | COMMON SOURCE |
Indol	M.R.	V.P.	Citrate	Usually	Occasionally	
−	+	−	−	*Bact. coli*	Non-members of group	Feces
+	+	−	−	*Bact. coli*	Very occasionally non-members of group	Feces
+	+	−	+	Mixture of *Bact. coli*, extraneous form or *Bact. aerogenes*	Intermediate strain	Soil
−	+	−	+	Intermediate strain	Mixtures or slow secondary reacting *Bact. acrogenes*	Soil (rarely feces)
+	+	+	+	Mixtures of *B. coli* or *B. aerogenes* with extraneous form	Atypical	Soil
+	−	−	+	Always mixtures, usually contain aerogenes, sometimes coli		
+	−	+	+	Mixtures of aerogenes with extraneous form, sometimes coli	*Bact. cloaçae*	Soil
−	+	+	+	Mixtures containing aerogenes	Atypical	Soil
−	−	+	+	*Bact. aerogenes*	Non-lactose fermenting. May or may not be spore forming	Soil (rarely feces)
−	−	−	+	Extraneous form not a member of group	*Bact. ae, ogenes*	Soil

This study of purification also indicated that all *Bact. coli* strains did not grow and some *Bact. aerogenes* strains did grow at 46°C. on the common laboratory media.

3. A careful study of 32 samples of human and animal feces was made. Two special methods of isolation for obtaining the

Fig. 3

Purification of Coli-Aerogenes Cultures
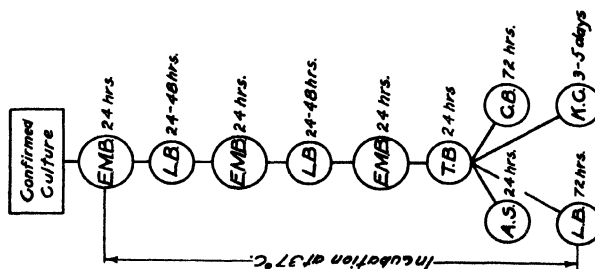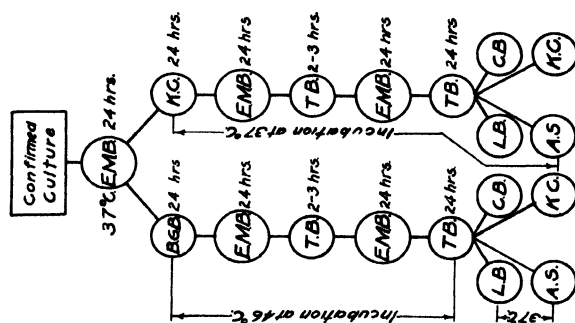
1-Common Method   2-Split Culture Method   3-Tryptophane Suspension Method   4-Pour Plate Method
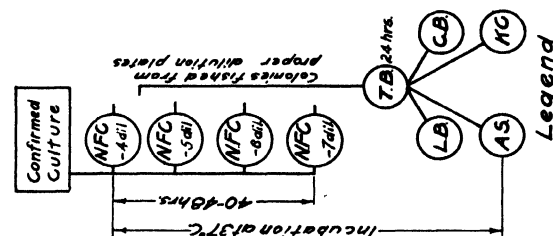
Legend

E.M.B. = Levines E.M.B. agar plates
N.F.C. = Noble's Ferrocyanide citrate agar
B.G.B. = green bile broth   C.B. = M.R.-V.P. med.
L.B. = lactose broth   K.C. = Koser's cit. sol.
T.B. = Tryptophane broth   A.S. = agar slant

minority type strains were used. With these methods 462 *Bact. coli* strains, 16 *Bact. aerogenes* and 10 of the $- + - +$ intermediate strains were isolated. None of the other intermediate or atypical strains that may be isolated from water or soil were obtained.

CONCLUSIONS

We conclude from these studies that differentiation of cultures from water obtained after preliminary enrichment in lactose broth is usually unsatisfactory. This is on account of unpredictable elements in the enrichment process, the difficulty of obtaining pure strain isolations and the complexity of the Coli-aerogenes group which requires the use of at least four differential tests for proper interpretation of results. Where exact differential results are necessary, the four tests studied in this paper should be applied to numbers of cultures obtained by direct planting of the samples into some solid medium. Such careful study is very valuable in judging the exact quality of a water when few samples are to be tested.

We also wish to make a plea for more careful study of cultures, including differentiation obtained during studies of the comparative value of various media in routine work. Owing to the manner in which such work is usually conducted at the present time it is impossible to tell the nature of the strains isolated by the standard method, to say nothing of the strains obtained by the new methods or media that are being studied. We believe that, with all of the data now available, improvements might be made in "Standard Methods." Such improvements should be toward an elimination of the deficiencies of the present method without sacrificing the yield of the coli-aerogenes group.

REFERENCES

BARDSLEY, D. A. 1926 Jour. Hyg., **25**, 11.

BAHLMAN, C., AND SOHN, H. 1924 Jour. Amer. Water Works Assoc., **11**, 416–33.

BEDFORD, R. H. 1929 Jour. Bacteriol., **18**, 93–4.

BERGEY, D. H. 1925 Determinative Bacteriology. Williams and Wilkins.

BLUMENBERG, W. 1928 Centr. Bakt. Para. I Abt., **107**, 386–92; Chem. Abs., **22**, 3473.

BROWN, J. W., AND SKINNER, C. E. 1930 Jour. Bacteriol., **20**, 139–150.

BUCHANAN, R. E., AND FULMER, E. I. 1930 Physiology and Biochemistry of Bacteria. Williams and Wilkins.

BUTCHER, B. H. 1926 Jour. Amer. Water Work Assoc., **15**, 171–73.

CHEN, C. C., AND RETTGER, L. F. 1920 Jour. Bacteriol., **5**, 253.

DAWSON, A. I. 1919 Ibid., **4**, 133.

DULANY, A. D. 1928 Jour. Inf. Dis., **42**, 575–88.

GEORGIA, F. R., AND MORALES, R. 1926 Jour. Amer. Water Works Assoc., **16**, 631–41.

GINTER, R. L. 1927 Ibid., **17**, 591–94.

HADLEY, PHILIP 1927 Jour. Inf. Dis., **40**, 1–388.

HICKS, E. P. 1927 Jour. Hyg. **26**, 357.

HOUSTON, A. C. 1911 Seventh Research Report of the Metropolitan Water Board, London, England.

HOUSTON, A. C. 1928 Twenty-third Annual Report of the Metropolitan Water Board, London, England.

HURWITZ, E., AND KRAUS, L. S. 1929 Eng. News-Rec. **102**, 704.

ISHIKAWA, M. 1927 Jour. Inf. Dis., **41**, 238–56.

JONES, H. N., AND WISE, L. E. 1926 Jour. Bacteriol., **11**, 359–66.

JORDAN, E. O. 1928 General Bacteriology. W. B. Saunders Company.

JORDON, H. E. 1927 Jour. Amer. Water Works Assoc., **18**, 337–46.

KLUYVER, A. J. AND DONKER, H. J. L. 1926 Chem. Zelle und Gewebe, **13**, 134–90.

KOSER, S. A. 1923 Jour. Bacteriol., **8**, 493–520.

KOSER, S. A. 1924 Jour. Bacteriol., **9**, 59–77.

KOSER, S. A. 1924 Jour. Inf. Dis., **35**, 14–22.

KOSER, S. A. 1925 Jour. Bacteriol., **11**, 409–16.

KOSER, S. A. 1926 Jour. Inf. Dis., **38**, 506–10.

KOSER, S. A. 1927 Amer. Jour. Pub. Health, **17**, 1178–82.

KOSER, S. A., AND SHINN, W. C. 1927 Jour. Amer. Water Works Assoc., **18**, 328–336.

KOVÁCS, N. 1928 Ztschr. f. Immunitätsforsch. u. Exper. Therap., **55**, 311–15; Chem. Abs., **22**, 3425.

LEITER, L. W. 1929 Amer. Jour. Hyg., **9**, 705–724.

LEVINE, MAX 1921 Bull. 62, Eng. Exp. Station, Ames, Iowa.

LEWIS, I. M., AND PITTMAN, E. E. 1928 Jour. Amer. Water Works Assoc., **19**, 78–92.

LINTON, C. S. 1924 Abs. Bacteriol., **8**, 295.

MASSINI, R. 1907 Arch. f. Hyg., **61**, 250.

MEYER, E. M. 1918 Jour. Bacteriol., **3**, 9–14.

NEISSER, M. 1906 Centralbl. f. Bakteriol., I, **38**, 98.

PAINE, F. S.   1927   Jour. Bacteriol., **13**, 269–74.

PAWAN, J. L.   1925   Ann. Trop. Med. and Parasitol., **19**, 319.

PERRY, C. A.   1929   Amer. Jour. Hyg., **10**, 580–613.

PRESCOTT, S. C., AND WINSLOW, C.-E.A.   1924   Elements of Water Bacteriology. John Wiley and Son.

RAGHAVACHARI, T. N. S.   1926   Ind. Jour. Med. Res. **14**, 47.

RUCHHOFT, C. C., KALLAS, J. G., AND CHINN, BEN   1931   Jour. Amer. Water Works. Assoc., **23**, 565.

SEARS, H. J., AND PUTTMAN, J. J.   1923   Jour. Inf. Dis., **32**, 270.

SIMMONS, J. S.   1926   Ibid., **39**, 209.

SOULE, M. H.   1928   Ibid., **42**, 93–148.

Standard Methods of Water Analysis.   1905, 1923, 1925   American Public Health Association.

TONNEY, F. O., AND NOBLE, R. E.   1930   Jour. Amer. Water Works Assoc., **22**, 488–501.

WERKMAN, C. H.   1930   Jour. Bacteriol., **20**, 121.

WERKMAN, C. H., AND GILLEN, G.   1931   Ibid., **21**, 6.

WILLIAMS, O. B., AND MORROW, M. B.   1928   Ibid., **16**, 43.

LIVING MICROÖRGANISMS IN ANCIENT ROCKS

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In September, 1928, I reported in a note in *Science* that I had discovered living microörganisms in pre-Cambrian and other old rocks. In April, 1930, I reported, also in *Science*, an item on artificial bacilli in connection with which I stated that the work on microörganisms in rocks was being continued but that anthracite coal was then receiving exclusive attention, studies on other rocks being held in abeyance for the present. In this statement I shall confine myself entirely to my researches on anthracite coal which have thus far yielded the most striking evidence obtained in any of my studies on the existence of living microörganisms in ancient rocks.

I have been asked frequently since the inception of the studies under consideration to state what led me to make such an investigation when *a priori* one would not expect anything but negative results. My answer to this question is that for twenty years prior to the initiation of these experiments I had been accumulating more and more evidence on the persistence of the life of bacteria and bacterial spores for periods of forty years, as a maximum in the latter case, as authentic facts. The unabated virulence of pathogenic organisms grown from very old spores and the remarkable viability of bacteria from very old and very dry soils preserved in unopened bottles for about forty years have furnished me with much food for thought for well nigh a quarter of a century. Circumstances conspired to prevent me from subjecting my hypotheses to experimental test until nearly four years ago. Beginning with the oldest rocks on the face of the earth, the pre-Cambrian, and finding some evidence of bacteria in a living condition in them, I turned to rocks of other periods, and finally to anthracite coal because its origin promised for it the existence of a larger if not more varied flora. I argued

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that if a spore or some other phase of a microörganism could persist alive for forty years there is no real reason why it might not do so for much longer periods even to millions of years.

Since the technique of such an investigation is the most vital factor in determining the validity of its results I shall refer in this and in another part of my paper, in a detailed manner, to the experimental procedures employed. At the beginning of the work on anthracite coal I used commercial coal samples derived both from Wales and from Pennsylvania. Later I used specially collected samples of anthracite obtained at a depth of 1800 feet in a mine near Pottsville, Pennsylvania. The general procedure was as follows: A piece of coal about 2 to 3 inches in diameter was thoroughly scrubbed with soap and hot water and rinsed in distilled water (hot and cold). It was then dried with a paper towel and submerged in a beaker of Superoxol (30 per cent solution of H_2O_2). After five or six hours of exposure in Superoxol, the coal was transferred by means of a sterilized and very hot pair of tongs to a beaker of 95 per cent alcohol. After a few seconds of submergence in the alcohol it was grasped by another pair of sterile and very hot tongs and enveloped in the flame of a large burner until the alcohol had burned off, and then thrust into a large cast-iron, thoroughly sterilized mortar. The mortar was completely covered, pestle and all, except at its base, before sterilization, by two or three layers of clean absorbent cotton tied tightly to the mortar. The whole was sterilized in a hot air oven for no less than two days (at times for five and six days) at a temperature of 160° to 170°C. The time of exposure of the coal to the air as it went almost red hot from the flame into the mortar was perhaps a second. A flap in the cotton was raised for the purpose of transferring the coal to the mortar and immediately tied down again. By grasping the cotton covered and protected pestle and pounding, it was possible to crush the coal to powder in a minute or two. The mortar was then moved to another part of the laboratory (in more recent experiments to a specially constructed inoculation chamber). By raising the flap of cotton again and inserting a

large, long-handled, sterile and almost red-hot spoon into the mortar, it was possible to obtain a spoonful of coal powder for a quick transfer to an Erlenmeyer flask of sterile medium or to sterile water if a dilution experiment was made. No glassware was used before it had been sterilized for several hours at about 160°C. No medium was used without sterilization at least twice in the autoclave at 20 pounds pressure for periods of one and a half to two hours. In addition, media received an extrasterilization for half an hour to an hour just before being used. The cotton stoppers (after the medium had received the coal powder) were covered immediately with a piece of filter paper soaked in a 1:1000 HgCl₂ solution and the paper was fastened under the lip of the flask. After different intervals of incubation, usually after three or four days, the cultures were streaked or plated on an agar medium and were examined directly under the microscope. As was stated a moment ago, the foregoing procedure was generally employed in preparing the cultures; but many modifications of some features of the technique were employed. Some of these modifications will be mentioned presently. It is to be noted that about 100 experiments were performed which furnish the basis for this report. Obviously it is not possible here to do anything more than give a general summary of these numerous experiments but the crucial features of them will all be discussed.

A number of different types of media were employed in these investigations. The normal basis for all of them was coal extract made by heating powdered coal with tap water in the autoclave for ten to twelve hours at 20 pounds pressure and filtering and resterilizing. In some experiments, just the coal extract thus prepared was used as a medium. In other cases it was made up with 1 per cent peptone (the medium most widely used) or with starch. Other media used were 5 per cent glucose coal extract, sea water, and Bristol's algal medium.

GENERAL ACCOUNT OF RESULTS

Not all the replicate cultures in most series yielded growth but in some series they did. The rapidity of the appearance of growth varied with the medium and with the manner in which the coal

had been treated, as well as with the source of the sample. In general, however, the peptone media yielded growth in twenty-four to seventy-two hours (in 1 case, in five hours). In coal extract alone, growth might not be detectable until after two or three weeks of incubation. The types of organisms obtained showed only a slight range of variation. Most of them were short bacilli or cocco-bacilli and egg-shaped coccus forms, varying considerably in size and shape even in pure cultures. The tendency among all of them to occur in pairs was very marked. The colonies they produced on agar media at the first streaking were usually small and bead-like. Some of them were yellow to yellowish brown, others white, but in thin layers of growth, a distinctly blue color was always visible. In addition there were the ubiquitous blue, shadowy, somewhat larger colonies deep in the agar. The yellow colonies developed frequently as large surface colonies but quite commonly also as lenticular or cockade-shape colonies of smaller size, from which larger colonies would often develop. In some of the organisms there is a marked difference in appearance between a culture a day or two old and one of the same pure type several weeks old as exemplified strikingly in some of the microphotographs accompanying this paper. Moreover, the variability within a young pure culture was frequently most marked as regards both shape and size of the organism. Thus far I have not been able to demonstrate the existence in anthracite coal of molds, yeasts, or algae, but the number of experiments require manifolding to obtain indications of finality with regard to this last question. Apparently all of these organisms produce visible or invisible spores, or resting stages which serve the same purposes as spores. My results give fresh support, in ways which I have not time here to describe, to the conviction which I have often voiced during the last twenty years or more that all bacteria produce spores or bodies serving the same purpose, and that the distinction between spore and non-spore bearing organisms, while perhaps useful in certain ways, is not based on any critical study of resistant or resting stages in all forms. Certainly I regard the assumption that coccus forms do not produce spores, or something serving the same purpose as

spores, as being gratuitous and out of harmony with many interesting observations.

In a continuation of my discussion of the results obtained with my cultures, I come to another phase of my experimentation which involves a somewhat different technique from that which I have described, and which is fascinating in its far-reaching possibilities as regards this problem. Before adopting Superoxol as a bactericide for the sterilization of the exterior of coal or other rock samples intended for crushing, I had spent months in studying the effectiveness of all kinds of bactericidal agents and found nearly all of them useless for my purposes for one reason or another. The most effective I found was Superoxol and I shall state below how I checked the effectiveness of Superoxol before using it extensively. Nevertheless, it occurred to me that if heating a rock sample could accomplish the sterilization of its surface without killing all the bacteria inside, such procedure might serve as a good check on the Superoxol treatment and at the same time, might yield other important results regarding the heat resistance of the organisms under study. I carried out, therefore, many experiments with coal heated for various periods, both in the autoclave at 20 pounds steam pressure (about 115°C.) and in the hot air oven at a temperature of 160° to 170°C., principally by the latter method. Periods of heating ranged from two and one-half to twenty hours in the autoclave and from five to fifty hours in the hot air oven. Again, my results are too numerous to be given in detail in this brief communication but they may be summarized as follows: The surface of anthracite coal may be sterilized in ten hours or more in the hot air oven and in the autoclave in five hours without killing all the organisms inside of the coal even though heat penetration studies with thermocouples on the coal have demonstrated that the interior of such samples as I used in the hot air oven attains the temperature of the oven in about two hours after the sample is placed in the oven. It is still uncertain how effective the heating in the autoclave may be in point of lethal time for the interior of the coal. In the case of coal heated in the hot air oven, however, it has proved impossible to kill all the bacteria in the interior of the coal

sample even in a period of fifty hours under the conditions described above. Longer periods have not yet been tried. Moreover, it seems as if the longer periods of heating cause the organisms to grow more effectively in the cultures prepared from the crushed coal subsequent to the heating than do the shorter periods of heating. There is something very remarkable about this phenomenon which leads to possible implications in my mind which I prefer not to discuss for the present.

CHECKING THE TECHNIQUE EMPLOYED

In appraising and interpreting the results obtained by inoculating sterile media with crushed coal whose surface has been sterilized either by chemicals or by high heat a number of questions naturally arise with reference to the adequacy of precautions against contamination. I shall take up each of these questions separately in order to show how they are eliminated from consideration as determining factors in the results obtained. First, contamination of cultures from atmospheric sources during the placement of a sample of coal into the sterile mortar and during the distribution of the powder made by crushing such a sample into sterile media.

1. A sample of coal was broken into pieces not longer than ½ inch and placed in a sterile wide-mouth Erlenmeyer flask, stoppered with a cotton stopper, and put into the oven which was run at a temperature of 160° to 170°C. for a little over six days. After the flask was cooled at the end of that period, the coal was quickly transferred to a sterile mortar, as in the case of the routine technique with other samples. It was crushed to powder and distributed into six flasks of sterile 1 per cent peptone coal extract medium. After three or four weeks, an examination of these flasks directly and by transfers to agar slants of the same medium, showed no growth whatever. This experiment was repeated and the cultures thus prepared were plated, used for inoculation of slants and directly examined microscopically. Except for one or two fungi which appeared as air contaminants in these six containers, no microörganisms were found.

2. To check still further the experiments just described, a

sample of quartz sand was heated in the oven at a temperature of 160° to 170°C. for four days, using the same technique as was used with the coal. It was then transferred to a mortar, ground
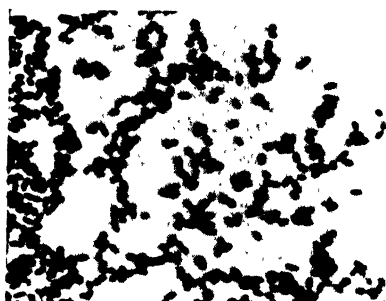
FIG. 1   FIG. 2
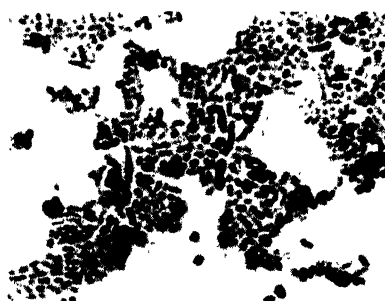
FIG. 1. FORTY-EIGHT-HOUR CULTURE ISOLATED FROM PENNSYLVANIA COAL, HEATED IN OVEN AT 160° TO 170°C. FOR FIFTEEN HOURS AND CRUSHED

Figures are in all cases magnified 1129 diameters
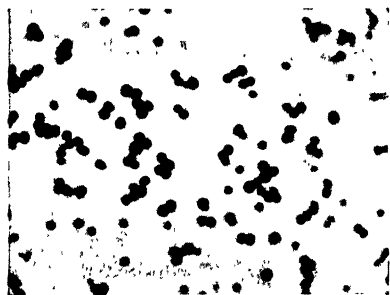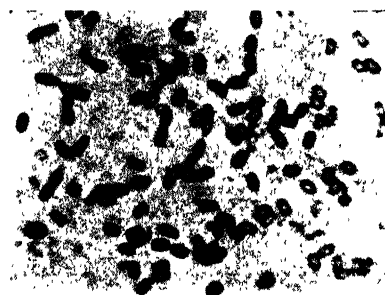
FIG. 2. SAME CULTURE THREE MONTHS OLD

FIG. 3   FIG. 4

FIG. 3. FORTY-EIGHT-HOUR CULTURE ISOLATED FROM PENNSYLVANIA COAL, HEATED TEN HOURS IN OVEN AT 160° TO 170°C. AND CRUSHED, AFTER STAYING IN THE UNCRUSHED CONDITION FOR THIRTY-NINE DAYS IN COAL EXTRACT PEPTONE MEDIUM, WITHOUT SHOWING GROWTH

FIG. 4. SAME CULTURE THREE MONTHS OLD

with a pestle to some extent and inoculated into six flasks of sterile medium. Even after two months, these cultures showed no growth whatever and the medium remained perfectly clear.

Direct examination and streaking on agar gave no evidence of organisms.

3. Five samples of commercial coal derived from both Wales and Pennsylvania, of about the same size as those used in the
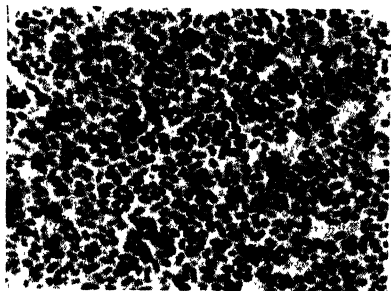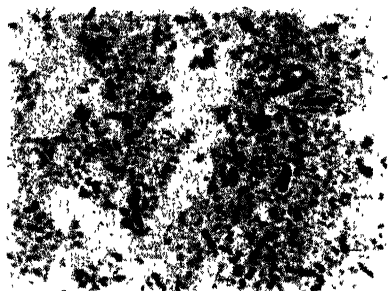
FIG. 5   FIG. 6

FIG. 5. FORTY-EIGHT-HOUR CULTURE ISOLATED FROM WALES COAL HEATED FOR FIFTEEN HOURS IN THE OVEN AT 160° TO 170°C. AND CRUSHED

FIG. 6. SAME CULTURE THREE MONTHS OLD
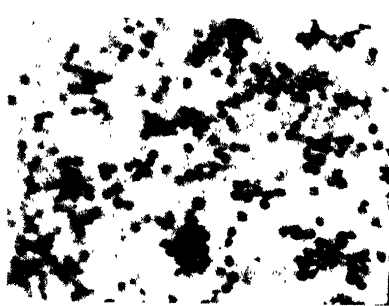
FIG. 7   FIG. 8

FIG. 7. FORTY-EIGHT-HOUR CULTURE ISOLATED FROM WALES COAL HEATED IN THE OVEN FOR FIVE HOURS AT 160° TO 170°C. AND CRUSHED, AFTER HAVING BEEN ALLOWED TO REMAIN IN AN UNCRUSHED CONDITION FOR 55 DAYS IN COAL EXTRACT PEPTONE MEDIUM, WITHOUT SHOWING GROWTH

FIG. 8. SAME CULTURE THREE MONTHS OLD

routine experiments described, were placed in Superoxol for periods varying from three to six hours, then transferred to alcohol. The sample was taken out of the alcohol, the alcohol was

burnt off, and the sample kept enveloped in the large flame for two or three seconds longer and quickly transferred to sterile peptone coal extract medium in a wide mouth Erlenmeyer flask. To protect the cotton stopper from dust, it was covered by a piece of filter paper soaked in a solution of $HgCl_2$, concentration 1:1000. After periods varying from one month to three months, it was found that the media into which these coal samples were placed remained clear and transfers from them showed no microorganisms. The different samples were then crushed as in the regular procedure which I have described, and distributed into
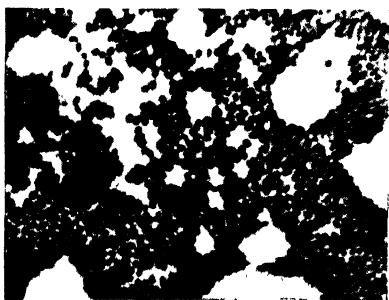
FIG. 9   FIG. 10
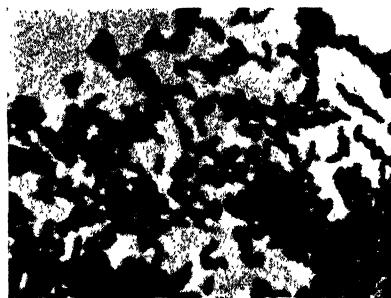
FIG 9. FORTY-EIGHT-HOUR CULTURE ISOLATED FROM WALES COAL AFTER COAL
WAS EXPOSED IN SUPEROXOL FOR FIVE HOURS AND FIFTEEN
MINUTES AND CRUSHED

FIG. 10. SAME CULTURE THREE MONTHS OLD

fresh sterile culture media. In the case of every piece of coal thus treated, the crushed material yielded growth in more than half of the cultures made from it. In some cases, growth was obtained in all of the cultures made from a given piece of coal.

4. Five samples of commercial coal derived from Wales and Pennsylvania were heated in wide mouth Erlenmeyer flasks in the hot air oven at temperatures varying between 160° to 170°C. for periods of five to ten hours. At the end of the period of heating used in each case, the flasks were cooled and sterile 1 per cent peptone coal extract medium was poured quickly over the coal samples, sufficient to submerge them. The cotton stopper

was then replaced, covered with a protecting filter paper soaked in $HgCl_2$ as before, and placed in the incubator as were the other samples.   After periods varying from one month to three months in the incubator, the samples were removed and it was found that the medium remained perfectly clear in all cases and an examination of the supernatant liquid, directly, and by means of slants, showed no microörganisms present.   The samples were then removed from the flasks, dipped into alcohol, the surface again burnt in the alcohol flame and additionally in the open flame of a large burner, and then transferred to a sterile mortar and crushed as in the case of the other series.   Sterile culture media inoculated with coal powder thus prepared gave growth in more than half of the several cultures made from each piece of coal.   In one or two cases, all of the cultures thus prepared showed growth.

The organisms found in the cultures described as prepared from pieces of coal whose surface only had been sterilized, either by chemicals or by heat, were limited to two or three forms closely similar, if not identical with the cocco-bacillus and very short diplo-bacillus which I have just described as being found quite uniformly in anthracite coal.   This applies to all coal samples examined, whether from commercial coal, or from the special samples obtained from a great depth in a coal mine in Pottsville, Pennsylvania.

5. As a further check on my technique, it seemed desirable that if possible, plate cultures with or without dilution be made immediately after a sample of coal was crushed, so that it might be possible to observe the production of colonies, if any, from the dormant cell forms in the coal.   This seemed particularly desirable since it has never been possible for me to observe directly in powdered coal any vegetative or even spore forms of microörganisms.   Twelve samples of coal, mostly derived from the heart of the special sample from Pennsylvania, were sterilized on the surface, in half of the cases with Superoxol and in the other half by heating ten hours in the oven at 160° to 170°C., crushed, transferred into thoroughly sterilized and filtered tap water, shaken for three to five minutes, and quantities varying from 1 to 2 cc. were distributed immediately into sterile Petri dishes and sterile

peptone agar poured over the suspensions in the plates.   In nearly all of these series which were so arranged as to be used in quantitative determinations of the numbers of microörganisms in the coal, it was found after the proper periods of incubation that no colonies developed; but after the coal suspension in tap water from which these dilutions were made had been incubated for a few days to three weeks, plates similarly made yielded colonies in nearly all cases.   It should be remarked that these colonies were produced from organisms which had developed in nothing but a suspension of coal powder in sterile tap water.   They had apparently multiplied enormously in that medium and yet the cells from which these numerous organisms were derived were incapable in agar of producing colonies in nearly all of the plates made in the original quantitative experiment.   Apparently the immediate transfer of a suspension from the coal powder in tap water to agar inhibits the development of the dormant cells into vegetative forms.   This striking phenomenon was checked further in some of the series of experiments in this category by transferring from 1 to 5 cc. of the coal-powder-tap-water suspension immediately after it was made, to liquid sterile media in Erlenmeyer flasks.   After a few days, in which the media at times remained clear in these new cultures, and at times became slightly turbid, plates poured from these transfer cultures always yielded colonies which in turn showed organisms of the same types as those described earlier in this paper.   This proved true when the most favorable medium was employed, namely, 1 per cent peptone coal extract, for the transfers from the original suspension, or when pure distilled-water-clear-coal-extract itself was used as a medium, or when 1 per cent peptone-starch-coal-extract was used as a medium.   Incidentally, the several series of plates which showed no colony growth serve as another check on my technique as regards the plating of any of the culture series.

DISCUSSION AND CONCLUSIONS

The literature available on studies of coal carried out in various ways does not, so far as my search has gone and this has admittedly not been complete, reveal any case of an investigator who

has indicated the possibility that microörganisms found in coal may be a few living relics of originally abundant flora in the material from which the coal was derived perhaps as long as one or two hundred million years ago. Most of the investigators who have concerned themselves with studies on the bacterial flora of coal do not even suggest that the organisms which they found in coal are anything more than modern bacteria which have come into the coal from the outside in very recent time. Such seems to be the case in the investigations of Schroeder, of Galle, and of Potter. On the other hand, Lieske, in a very brief note discussing his investigations, dismisses with a word the suggestion that the microörganisms which he found in coal and which he regarded as belonging to the species *Bacterium liquefaciens-fluorescens* and perhaps one additional form, could possibly be representatives of any ancient bacteria which have lived over in the coal from the time of its formation. In other words, therefore, I am making a claim which so far as I know, has never been made in connection with the studies of bacterial flora of rocks, (practically all of these have been limited to coal, and there have not been many) namely, that the microörganisms found in coal are actually survivors, imprisoned in the coal at the time it was formed, from material which originally was probably very rich in microörganisms since it was peat-like in nature. It is my view that here and there scattered through the masses of the coal measures an occasional spore or some similarly resistant resting stage of a microörganism has survived the vicissitudes of time and circumstance and retained its living character, its power to develop into a vegetative form, and its power to multiply when conditions are rendered propitious for it. I do not regard it as at all necessary that a living cell like a spore destroy itself by respiring away its own substance in the course of time. I believe that it is quite possible for a cell like a spore to remain in a state of suspended animation with respiration not occurring at all, and I believe that in a thoroughly desiccated condition of its protoplasm as would be the case with a spore locked up in coal, that a state of suspended animation is quite a conceivable and even probable phenomenon in nature. If one assumes such a hypothesis and

further, the hypothesis that spores have survived only in small numbers and occur sporadically within the coal mass or in the mass of other rocks, then one can readily see how results could be obtained similar to those which I have obtained in some one hundred experiments which I have carried out with anthracite coal.

From the studies which I have made of the literature which bears on the observation of microörganisms in coal, I feel that no investigator besides myself has really taken the precautions necessary to a proper study of microörganisms of coal, if the question of their having lived over from the time of the formation of the coal is held in view. I have employed such rigorous methods for preventing contamination that I have felt at times that there was a possibility that some organisms which were present in the coal samples used were actually destroyed by the drastic methods which I used for the sterilization of coal surfaces and of apparatus.

Before I close, I desire to discuss one other factor which bears on the validity of my conclusion. Anyone who has thought about a problem of this sort must necessarily have surmised that organisms found in anthracite coal today, and perhaps also in other rocks, might have gained entrance into such rocks very recently through crevices or pores which the rocks might contain. In fact, Lieske has expressed the view that the organisms which he found in anthracite coal were washed down into the coal measures from the surface of the ground which covers the coal, by seepage water and other surface water percolating downward. In the course of my experiments, therefore, I addressed myself to studies or to special precautions which might resolve that difficulty. As regards precautions, I may say that after working for a long time with commercial coal, I had collected for my use specially large blocks of coal as above stated from a deep mine near Pottsville, Pennsylvania. The gentleman who was kind enough to collect these samples for me, Mr. Raymond C. Johnson, Research Chemist of the Philadelphia and Reading Coal and Iron Company, assures me that the samples were collected from a place in the mine at a depth of 1800 feet from the surface, where there is no

evidence whatever of percolating water in or near the point at which the samples were obtained, and yet cultures made from this coal give results practically identical with those made from commercial samples of coal. Such evidence, however, may not be as satisfying as direct studies upon the penetrability of coal samples by chemical substances and by living cells. I therefore made a study of the permeability of coal to the following substances: water, sodium chloride, in solution, mercuric chloride in solution, potassium dichromate in solution, and eosin. I used these substances because it was possible to employ a test for each of them after the coal had been submerged in the solution in question. The tests were made by submerging carefully washed chunks of coal in these substances and keeping them in that submerged state for one to three weeks. I found that all of these substances penetrate coal fairly readily and particularly in certain areas of the coal which seem to be more permeable than others. Owing to the limitation of time I shall not go into a discussion of the tests used, especially in view of the fact that they all showed positive penetration of these substances into the coal. It must be remarked, however, that these substances are composed of molecules which even in the case of the largest of them, namely the eosin molecule, are very small compared to bacterial cells, and for that reason I started an experiment to determine whether or not a sample of coal which had been thoroughly heated and sterilized could be penetrated by bacteria from a suspension of the organism in a proper medium. The description of this experiment is as follows:

Two pieces of coal were carefully washed with soap and water and thoroughly rinsed in distilled water, and each placed in a wide-mouth Erlenmeyer flask of 500 cc. capacity. The flasks were stoppered with cotton and placed in the oven at 160° to 170°C., and kept there for six days. At the end of that time the flasks were taken out of the oven and allowed to cool, and sterile 1 per cent peptone coal extract was poured into one of the flasks sufficient to submerge the coal sample. Into the other flask a suspension of a coccus isolated from one of the coal samples studied earlier in the same medium was poured over the coal

sample.   Filter paper soaked in a solution of HgCl₂ of 1:1000 concentration was then adjusted over the cotton stopper and tied tightly under the lips of the flasks so that any dust or organisms would be prevented from touching the stopper.   The flasks were then placed in the incubator at 28°C. and kept there for over three months.   At the end of that time the coal sample which had been submerged in sterile peptone coal extract and which latter had remained perfectly clear and uncontaminated throughout that period, was removed to a sterile mortar, after dipping in alcohol and burning of the surface, and crushed.   The coal powder thus produced was inoculated into several flasks of sterile peptone coal extract medium.   Except for two or three organisms which were clearly air contaminants found in these cultures, they remained perfectly clear and streaking and plating from them yielded entirely negative results.

In the case of the sample of coal which had been submerged in a suspension of a pure culture of the coccus derived from coal, the following procedure was used: It was removed from the medium which was of course turbid, owing to the growth of the coccus, thoroughly rinsed in distilled water, dried, and placed in Superoxol for six hours.   At the end of that time, it was removed from the Superoxol, placed in alcohol for a minute, removed from the alcohol again and enveloped in a large gas flame until the alcohol had burnt off, and perhaps for a second or two beyond that time. The sample was then quickly placed in a sterile mortar and crushed, and the powder was distributed into several flasks of sterile peptone coal extract medium.   Only one of the several flasks produced any growth, and that after several days of incubation.   The growth was a contaminant which bore no resemblance to the coccus in a culture of which that coal sample had been submerged for three months.   The other flasks remained clear and after some time, were all plated on peptone coal extract agar.   A few colonies all told, perhaps not more than eight or ten, were found on these several plates.   It is obvious that if the coccus in which the coal sample was submerged had penetrated to any extent at all into the coal that each culture made from the crushed sample would have shown heavy growth and each plate

made from these cultures would have shown colonies too numerous to count.

The conclusion seems irresistible therefore, that particles as big as a coccus are too large to penetrate the coal, either through crevices or through microscopic pores as do the chemical substances which I discussed above.

The idea of Lieske, therefore, that organisms from surface water could have penetrated into anthracite coal seems to me to be untenable.   In view of this, I return to the conclusion which I presented above, namely that the microörganisms which I found in the anthracite coal are descendants directly from cells which have lain dormant there from the time of the coal's formation, which according to one method of the geologist's reckoning, would be fifteen million years, and according to another method, from one to two hundred million years.

FACTORS IN THE PREPARATION OF BACTERIO-PHAGE[1]

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Attempts to prepare large quantities of bacteriophage in this laboratory for therapeutic use have led to an observation considered worthy of reporting. The method of preparation generally'employed consists of inoculating young cultures into broth in the presence of an inciting medium, followed by brief incubation at 37°C. and filtration—the process being repeated until bacteriophage of the desired potency is obtained. This procedure proved successful in our hands in preparing small amounts of bacteriophage such as 50 cc. In attempting to apply it to the preparation of larger amounts, several liters for example, difficulties were encountered, the products showing wide variation in lytic powers and often a tendency to give rise to secondary growth. After some trial, a satisfactory method for preparing large quantities of bacteriophage was developed, and its basis and technique will be considered in the present article.

The method is based upon an observation that staphylococcus bacteriophage inoculated with staphylococci increased in potency after prolonged incubation at room temperature. This increase occurred even when resistant forms developed during incubation contrary to the general assumption that such forms reduce bacteriophage potency by adsorption of the lytic principle. The observation was first made in an experiment with bacteriophage prepared with Strain H which was obtained from Dr. André Gratia of the Pasteur Institute, Brussels. A series of broth-dilutions of this bacteriophage, ranging from 10^{-1} to 10^{-10}, were inoculated

with staphylococci and incubated at room temperature. As is often the case, some of the dilutions gave rise to secondary resistant forms. The individual dilutions were filtered after seven days' incubation at room temperature and tested for lytic power. It was found that the filtrates of these dilutions possessed higher lytic power than the original bacteriophage from which the dilutions were made. The increase in lytic power was found even in case of dilutions 10^{-1}, 10^{-2}, and 10^{-6}, which had contained resistant forms. This observation suggested that, under certain conditions, prolonged room temperature incubation enhances the lytic power of bacteriophage in spite of the presence of resistant forms.

The results of the same experiment indicated that prolonged incubation in the production of bacteriophage exerted another effect of practical importance. Thus, the original lot of bacteriophage from which the dilutions were made began to show clouding with secondary growth after three weeks at room temperature, while the filtrates of the dilutions remained clear for over five months at the same temperature, when they were discarded. It appeared from this finding that prolonged room-temperature incubation tended to produce bacteriophage filtrates which were relatively "stable," remaining clear and free from secondary growth.

METHOD FOR PREPARING LARGE QUANTITIES OF BACTERIOPHAGE

In applying the foregoing observations to the preparation of four- and six-liter amounts of bacteriophage, often required for therapeutic use, the following method has been developed.

The method has two prerequisites. A bacteriophage having a lytic titer[2] of 10^{-6} or higher, and an inoculum prepared as follows:

[2] In this work the lytic titer was ascertained as follows: eleven test-tubes of 17-mm. diameter containing 8 cc. of broth, pH 7.6, were inoculated with 1 loop of a six-hour culture of the organism and incubated for six hours at 37°C. To ten of the cultures bacteriophage was added to give dilutions of 10^{-1}, 10^{-2}, and so on through 10^{-10}. The eleventh culture was left without the addition of bacteriophage, as a control on the inoculation. To a twelfth tube of 8 cc. broth, uninoculated, was added 0.8 cc. of bacteriophage as a bacteriophage-control. The lytic titer was taken as the smallest amount of bacteriophage which determined complete lysis at the end of a seven-day period.

(a) A loop of the homologous organisms is inoculated into a tube of broth (about 6 cc.), pH 7.6, and incubated for six hours at 37°C. (b) One part of this culture is added to a thousand parts of broth and incubated one hour at 37°C. This one-hour culture is referred to as the inoculum in the following outline.

1. To 100 cc. of bacteriophage is added an equal amount of inoculum and the mixture is incubated for twenty-four hours at room temperature.

2. If the mixture then shows no turbidity, 200 cc. of inoculum is added to it, and again incubated for twenty-four hours at room temperature.

3. If this mixture also is clear, an equal amount (400 cc.) of inoculum is added and incubated again for twenty-four hours at room temperature. This "doubling" procedure is continued until the desired volume of bacteriophage is obtained.

4. At this point, approximately 100 cc. of a six-hour broth culture per liter of mixture is added and further incubated for two days at room temperature. The mixture should now appear opalescent. If it is turbid, it should be filtered through a Berkefeld candle and the clear filtrate reinoculated with less than 100 cc. of a six-hour culture; if it is clear, an amount larger than 100 cc. of culture is employed for the inoculation. The aim is to add an amount of culture that will produce an opalescent mixture within two days of incubation at room temperature. This step is based on an observation that bacteriophage filtrates remained clear if, before final filtration, they had given rise to an opalescent or a flocculent form of resistant growth which was permitted to remain in the bacteriophage for some days at room temperature.

5. Opalescence having been attained, the mixture is incubated at room temperature for five to seven days, after which period it is filtered.

6. The clear filtrate is checked for lytic potency and for possible contaminations, and if satisfactory is ready for therapeutic use.

Special conditions. If after step 1 the mixture shows opalescence, it is incubated without filtration for about seven days to increase the titer, after which period it is filtered and the process continued with step 2. If after step 1 the mixture is turbid, indicating relatively low lytic potency, it is filtered after four days' incubation at room temperature, and an inoculum equal to half the volume of mixture, instead of the total volume, is added and incubated for twenty-four hours; if this mixture remains

clear, the process is continued with step 2; if opalescent or turbid, the steps are repeated as given in this paragraph.

In addition to the use of this method in increasing the volume of bacteriophage already prepared, it has also been employed, with slight modification, in preparing bacteriophage. Lytic principles were prepared for five staphylococcus cultures isolated directly from infections and found resistant to Gratia bacteriophage. The inciting medium used was either filtered pus or Gratia bacteriophage. The incubations were carried out for five days at room temperature. After this period all the final products showed bacteriophage action, the lytic titers ranging from 10^{-6} to 10^{-8}. A control incubated for five days was carried out similarly except that after each twelve-hour period of incubation the mixture was filtered and reinoculated; this method failed to produce bacteriophage for two of the five organisms. Another control, in which the twelve-hour incubation periods were carried out at 37°C., failed to produce bacteriophage for three of the organisms.

This method was also used in preparing bacteriophage for six strains of B. coli from urinary infections. The inciting medium was either sewage or colon bacteriophage which had been found to be inactive for these strains according to the ordinary tests. Incubation was carried out for five days at room temperature and all the final products showed lytic action ranging from 10^{-5} to 10^{-8}. Controls as above in which the individual incubation periods were limited to twelve hours were employed. The two control methods, however, failed to produce bacteriophage for two of the colon strains. It is of interest to mention that we have obtained one colon strain from a urinary infection for which we have not as yet succeeded in preparing bacteriophage with any of these methods. This strain is still under investigation.

ILLUSTRATIVE PROTOCOLS

In view of the numerous steps involved in preparing bacteriophage, especially in large amounts, it seemed well to present two illustrative protocols, one dealing with the preparation of bacteriophage employing the same agent as an inciting medium and the other employing sewage as an incitant.

Protocol 1.   Preparation of a four-liter amount of bacteriophage (lot 8) starting with 200 cc.

March 2, 1930.   To 200 cc. of staphylococcus bacteriophage, showing a lytic titer of 10^{-6}, was added 200 cc. of a one-hour inoculum (made by adding 1 cc. of a six-hour culture of staphylococci to 200 cc. broth of pH 7.6 and incubating at 37°C. for one hour).   The mixture was incubated at room temperature for twenty-four hours.

March 3.   To the 400 cc. of inoculated bacteriophage, which had remained clear, was added 400 cc. of a one-hour inoculum (made by adding 2 cc. of a six-hour culture to 400 cc. broth and incubating at 37°C. for one hour) and the mixture incubated at room temperature for twenty-four hours.

March 4.   To the 800 cc. of inoculated bacteriophage, which had remained clear, was added 800 cc. of a one-hour inoculum (prepared in the same proportions and with the same incubation conditions as above), and the mixture incubated for twenty-four hours at room temperature.

March 5.   To the 1600 cc. of inoculated bacteriophage, which had remained clear, was added 1600 cc. of a one-hour inoculum, and the mixture was incubated for twenty-four hours at room temperature.

March 6.   From the 3200 cc. of inoculated bacteriophage, which had remained clear, 150 cc. was removed (designated as control A) to be kept at room temperature in order to ascertain whether it would remain clear and free from secondary growth.

To the 3050 cc. of clear bacteriophage was added 1100 cc. of a six-hour inoculum, with the aim of producing an opalescent resistant growth. (The purpose of this step is presented in the text.)   The inoculated bacteriophage was incubated at room temperature for forty-eight hours.

March 8.   The bacteriophage showed opalescence, which was first visible after twenty-six hours of incubation.   An amount of 150 cc. was removed, for Control B, filtered and kept at room temperature to ascertain whether it would remain clear.

The residual 4000 cc. of opalescent bacteriophage was incubated at room temperature for five more days.

March 13.   The 4000 cc. of bacteriophage had remained opalescent and was filtered.   The filtered product was incubated for three days at 37°C. as a preliminary test for contaminations.

March 16.   The 4000 cc. of bacteriophage having remained clear, tests were set up for titration of potency; and for "sterility" in accordance with the requirements of the Hygienic Laboratory for biologicals intended for injection.

Control B had become cloudy, and a study of the growth occurring there revealed minute forms which produced smooth colonies 0.7 to 1.2 mm. in diameter. These gave, after three serial transfers on agar, opaque, white colonies 2 mm. in diameter and consisting of staphylococci. Both the minute forms and the staphylococcic forms were resistant to the bacteriophage from which the minute forms had been isolated.

March 20. Control A showed clouding, after fourteen days' incubation at room temperature.

March 23. Completion of the "sterility" tests showed no growth on laboratory mediums and no toxicity for guinea pigs. The titration tests showed a lytic titer of 10^{-6}. The 4000 cc. of bacteriophage was then stored at icebox temperature. Experience having demonstrated that with this method of preparation the bacteriophage shows no tendency toward clouding, this lot was ready for therapeutic use.

A small quantity of this lot was placed at room temperature with a view to ascertain whether prolonged incubation might produce clouding.

July 26. At the time of writing, after four months' incubation at icebox and room temperatures, this lot of bacteriophage is still clear.

Protocol 2. Preparation of a four-liter amount of bacteriophage (lot C48) employing sewage as inciting medium

March 4, 1930. To 100 cc. sewage filtrate was added 200 cc. of a one-hour inoculum prepared by inoculating 200 cc. of broth (pH 7.6) with 1 cc. of a six-hour culture of *B. coli* (Strain C48— a strain found to be resistant to bacteriophage preparation). The inoculated sewage was placed at room temperature to be incubated for five days.

A similar amount of inoculated sewage, designated control A, was prepared, to be filtered after twelve-hour incubation at the same temperature. Control B was prepared in the same way, to be filtered after twelve-hour incubation at 37°C.

March 5. Controls A and B were filtered and each inoculated with 1 cc. of a six-hour culture of *B. coli.* These controls were then incubated for twelve hours, control A at room temperature and control B at 37°C. After this incubation period each control was refiltered, reinoculated from a fresh six-hour culture, and again incubated at the designated temperature for twelve hours.

March 6. The process with controls A and B was repeated twice at twelve-hour intervals as on preceding day, with no evidence as yet of bacteriophage action against the colon bacillus.

March 7. The process with controls A and B was repeated twice again as on the two preceding days. Tests were set up with solid and liquid medium to ascertain whether the two filtrates possessed lytic action.

March 9. No evidence of lytic action was observed in the controls. The entire procedure was repeated.

The sewage culture that had undergone uninterrupted incubation at room temperature for five days was filtered and titrated for lytic action.

March 10. No evidence of lytic action was observed in controls. Entire procedure was repeated.

March 16. The lytic titer of the filtrate which had been incubated for five days before filtration was found to read 10^{-7}.

March 17. The control filtrates, A and B, both failed to show bacteriophage action; and since the period of time given to these control methods had already exceeded that required for obtaining bacteriophage by prolonged incubation, the control experiments were considered completed.

March 18. Having succeeded in the preparation of 300 cc. colon bacteriophage for strain C48, using sewage as an inciting medium, we next undertook to increase the volume to about four liters. Of the 300 cc. amount, 100 cc. was stored for reserve. To the 200 cc. remaining was added 200 cc. of a one-hour inoculum (made by adding 1 cc. of a six-hour culture of strain C48 to 200 cc. broth and incubating at 37°C. for one hour). The mixture was incubated at room temperature for twenty-four hours.

March 19. To the 400 cc. of inoculated bacteriophage, which remained clear, was added 400 cc. of a one-hour inoculum (made by adding 2 cc. of a six-hour culture to 400 cc. broth and incubating at 37°C. for one hour) and the mixture was incubated at room temperature for twenty-four hours.

March 20. To the 800 cc. of inoculated bacteriophage, which remained clear, was added 800 cc. of a one-hour inoculum (prepared in the same proportions and with the same incubation conditions as above) and the mixture was incubated at room temperature for twenty-four hours.

March 21. To the 1600 cc. of inoculated bacteriophage, which remained clear, 1600 cc. of a one-hour inoculum was added, with incubation for twenty-four hours at room temperature.

March 22. From 3200 cc. of inoculated bacteriophage, which remained clear, 150 cc. was removed (designated as control C) to be kept

at room temperature to ascertain whether it would remain clear. To the residual 3050 cc. of clear bacteriophage was added 1100 cc. of a six-hour inoculum, for the purpose of producing an opalescent resistant growth. The inoculated bacteriophage was incubated at room temperature for forty-eight hours.

March 24. The bacteriophage showed opalescence, which was first visible after twenty-six hours of incubation. One hundred and fifty cubic centimeters was removed, as control D, filtered and kept at room temperature to ascertain whether it would remain clear. The residual 4000 cc. of opalescent bacteriophage was incubated at room temperature for five more days.

March 29. The 4000 cc. of bacteriophage had remained opalescent and was filtered. It was incubated for three days at 37°C. as a preliminary test for "sterility." Controls C and D had become cloudy, and a study of the growth occurring in each revealed a mixed culture of minute colonies and of smooth colonies 2.5 to 3.5 mm. in diameter. Both types of colonies gave, after three serial transfers on agar, opaque, grey, flat colonies 4 mm. in diameter and consisting of gram negative rods. These forms were resistant to the bacteriophage.

April 1. The 4000 cc. bacteriophage having remained clear, tests were set up for titration and for "sterility."

April 8. Reading of the titration test gave lytic potency of 10^{-8}. The results of the "sterility" tests having complied with the Hygienic Laboratory requirements of biologicals for injection, the 4000 cc. of bacteriophage was stored for therapeutic use.

July 26. At the time of writing, after more than three months' incubation at icebox and room temperatures, this lot of bacteriophage is still clear.

<div align="center">COMMENT</div>

With the exception of one instance (lot 9) to be considered below, all attempts to prepare bacteriophage by the method outlined resulted in products which combined relatively high lytic potency with a tendency to remain free from secondary growth. A total of eleven lots of bacteriophage for staphylococcus and six for *B. coli* were prepared. The potency of these ranged between 10^{-6} and 10^{-9}. Ten of the lots were made to a volume of four liters, and seven were prepared in six-liter amounts.

In each instance when bacteriophage was prepared by the new method, as in the experiments represented by the foregoing protocols, samples were removed as controls at each phase of the process and passed through various modifications of the procedure. Some consisted of filtrations after various intervals shorter and longer than the five-day incubation period of the method described; others consisted of filtrations after incubation periods at 37°C. shorter and longer than five days. None of these modifications produced lytic filtrates as uniformly satisfactory as those resulting from this method.

The following observations were made regarding staphylococcus bacteriophage lot 9, which behaved differently from the others although prepared according to the same method. After the inoculation of 1100 cc. of a six-hour culture into 3 liters of clear bacteriophage with the aim of producing a resistant form of growth, the opalescent growth which was noted after thirty-nine hours of incubation at room temperature disappeared on the fourth day, instead of remaining apparently unchanged throughout the incubation-period as was the case in the other preparations. The incubation was continued, in accordance with the prescribed procedure, to be carried on for seven days. But upon a recurrence of resistant growth on the sixth day, this time in the form of a slight granular sediment, it was found necessary to extend the incubation period for twenty-nine days longer, because samples removed from time to time and filtered during this incubation period showed a tendency toward clouding after filtration. After the final filtration, however, following the twenty-nine days of incubation, the bacteriophage showed a lytic titer of 10^{-6} and has remained clear to date (three months). With the exception of lot 9, all the bacteriophage preparations followed the regular course. It appears that prolonged incubation in preparing bacteriophage leads to comparatively high lytic titer and relative permanence in clarity of the resulting filtrate.

SUMMARY

A method is described for preparing bacteriophage in large quantities for use in therapy. The resulting bacteriophage is of

relatively high potency and tends to remain free from secondary growth.   The outstanding characteristic of the method is the use of prolonged incubation at room temperature.   An outline of the procedure with illustrative protocols is presented.

I desire to take the opportunity to express gratitude to Doctor R. L. Kahn and Doctor Philip Hadley for their generous and invaluable counsel.

INHIBITION OF THE ACETONE-BUTYL ALCOHOL FERMENTATION BY ACIDS

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INTRODUCTION

During the course of a study of the effects of acids on the metabolism of the anaerobic organism *Clostridium acetobutylicum* (Weizmann) certain observations were made having to do with the more purely physico-chemical relationships of the acid association.[1] It is with some of these that this paper deals. Reference will be made to acid concentrations causing inhibition of the fermentation with considerations of the mechanism of the inhibitory effects.

An enormous literature has accumulated during the last forty years pertaining to the behaviour of microörganisms in acid media; since the early work of Kitasato (1888) and of Paul and Krönig (1896) the subject has been one of more or less continuous interest. With the general adoption, some fifteen years ago, of precise methods for the measurement of hydrogen ion concentration, microbiologists were enabled to characterize, much more definitely than was hitherto possible, the effects of many acids in terms of C_{H_+}. But, as is frequently the case at a time when an easily applicable method becomes available for the accurate estimation of a factor formerly capable of only approximate determination, one discerns in some of the literature of the period immediately

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[1] Until recently it has been the custom in this laboratory to name the organism *Bacillus granulobacter-pectinovorum*; but, in order to minimize confusion, the name suggested by McCoy, Fred, Peterson and Hastings (1926) has been adopted in this paper. The strain of the organism employed throughout all of our investigations is the original strain which was utilized industrially in Toronto during the war.

following the general introduction of pH measurements a tendency to emphasize unduly the significance of the information provided by the new technique. It seems to be generally recognized today, however, that the toxicity of acids for bacteria is not always merely a function of hydrogen-ion concentration, though in many cases this factor does, in reality, exert a predominant influence. Other factors involving, on the one hand, the anion, the undissociated acid and specific groups and, on the other, the nature of the organism and its substrate, must enter into any appraisal of the facts observed in studies of acid tolerance.

It is not proposed to review the extensive literature relating to the limits of acid concentration tolerated by microörganisms and to the general problems of the effects of acids on living cells. Summaries of investigations in these fields have been presented by numerous workers in the past. Attention is directed to certain papers which, in addition to contributing to the development of the subject, provide the key to the literature: e.g., Foster (1921), Wolf and Shunk (1921), Hall and Fraser (1922), Evans (1922), Berridge (1924), Pratt (1924), Falk and Harrison (1926), Katagiri (1926), Eggerth (1927). Investigations of a closely related nature, in the field of general physiology, have been reported by numerous workers, including Paine (1911), Harvey (1915), Crozier (1916, 1918), Collett (1919), Pantin (1923), Smith (1925), Lillie (1926, 1927), Velluz (1927), Taylor (1928), Bodansky (1928), Borissovsky and Wwedensky (1930). Some aspects of the problems of toxicity are presented by Miller (1920) and by Falk and Winslow (1926) while more extended reviews of related subjects are to be found in the works of Overton (1901), Traube (1913), Loeb (1913), Lillie (1923), Jacobs (1924), Henderson (1930).

The literature of the acetone-butyl alcohol fermentation reveals only a few references to studies of the growth of the related organisms in acidified media. None of the occasional studies which are encountered appears to represent a systematic investigation of the effect of any considerable range of acids. As early as 1876 Fitz observed that the growth of his butyl alcohol producing organism, *Bacillus butylicus*, in a medium otherwise favourable was impeded by 0.1 per cent (1.1×10^{-2} M) butyric acid. This

is an isolated observation and is of historical interest only, especially because the organism with which Fitz worked, though related to *Clostridium acetobutylicum*, was probably quite a distinct species, exhibiting a different degree of tolerance to acids and yielding different end-products. According to Fitz, his organism was quite acid-sensitive and it was necessary to culture it in the presence of $CaCO_3$. In Beijerinck's classical paper (1893) it is stated that "butyl bacteria are very sensitive to acids, 2 to 3 cc. of normal acid in 100 cc. being sufficient to stop the butyl fermentation completely." Here again the species studied were probably *not* identical with our organism. More recently Speakman (1920), working with *Cl. acetobutylicum*, observed that the fermentation proceeded to completion in cultures initially acidified with appreciable quantities of acetic, propionic and butyric acids. Limiting concentrations of these acids were not determined. At about the same time, Reilly *et al.* (1920) investigated the possible conversion of acetic acid to acetone when the acid was added to fermenting maize mash, the organism in this case in all probability being identical with our own. These workers however made no attempt to define the inhibiting concentrations of the acid. Fred, Peterson and Mulvania (1926) studied the influence of varying amounts of inorganic and organic acids on *"Granulobacter pectinovorum,"* an organism which is probably identical with *Clostridium acetobutylicum*. Qualitative observations were made on the influence of lactic, acetic, butyric, sulphuric, hydrochloric and phosphoric acids on growth, on gas production and on the formation of the characteristic "head" in 5 per cent maize mash. The general conclusion of these authors was that "it is not the percentage of acid, but the pH value which determines its inhibiting effect on the butyl alcohol fermentation." In the case of the six acids studied, observations indicated that growth and fermentation were severely retarded in flasks in which the initial acidity corresponds to pH values of 4.7 to 4.8 and that by neutralizing the acid this inhibiting property was removed. A preliminary abstract report of some of the results included in the present paper was made by the author some time ago (Wynne 1929).

These few papers appear to constitute the immediate literature

of the subject. It is here proposed to attempt to define more accurately than heretofore some of the physico-chemical relationships of the acid association and to discuss the mechanism of the inhibitory effects of acids.

In all work of this type distinction must be made between effects on growth and on fermentation. The two processes are, of course, not identical. There can be no appreciable fermentation without growth unless a relatively large number of organisms is added as inoculum. With maize as substrate it is very difficult to arrive at any quantitative estimate of growth. Ordinary anaerobic plating methods for determining numbers of cells are unreliable with this organism; direct counts have equally doubtful value, particularly in the case of maize cultures. Even with "liquid" cultures direct counts of this organism are frequently nearly valueless, owing chiefly to two factors: (1) the particularly slimy nature of the culture, at certain periods of its development, renders accurately representative sampling very difficult; (2) the organism characteristically exhibits peculiar aggregations the disintegration of which for purposes of direct counting is nearly impossible. Growth in liquid media such as glucose-peptone can, with a reasonable degree of accuracy, be defined positively or negatively upon careful observation of the culture. Quantitative estimates of numbers of organisms are best made, in such cases, with the assistance of nephelometric determinations of turbidity and comparison with known suspensions, or by estimation of the organisms precipitated by centrifuging a known volume of medium. Our observations on the growth of the organism in glucose-peptone solutions lead to the conclusion that the degree of fermentation runs parallel with the multiplication of the organism. But, though growth is often difficult to estimate accurately, degree of fermentation, on the other hand, can much more readily be put on a quantitative basis.

As applied to the experimental results which follow, the expression "inhibiting concentration" defines the degree of acidity, total and dissociated, which, when asociated with an otherwise favour-

able culture medium, is just sufficient to prevent completely the fermentation of 3 per cent maize by the organism at 37 to 38°C. To determine the acid concentrations which are capable of such effect we have employed a method involving the estimation of the total gas evolved during a given period of incubation. By this method the gas produced during the fermentation of small cultures containing, for example, 5 to 7 grams of carbohydrate in 3 per cent concentration was estimated by measuring the loss in weight of the cultures when the evolved gases were made to pass through concentrated H_2SO_4 contained in Alwood valves attached to the flasks. It was assumed that CO_2 and H_2 are the only gaseous or volatile products of the fermentation which are not absorbed by the acid. Total acidity was defined by titration; hydrogen-ion concentration was measured electrometrically, the readings being corrected to 25°C. for both the hydrogen and saturated KCl-calomel electrodes.

The substrate employed throughout the investigation was 3 per cent maize mash, prepared in the following manner. Portions of ground whole corn weighing 6 grams were suspended in 150 cc. distilled water in 300 cc. Erlenmeyer flasks and steamed at 100°C. for forty minutes, after which 50 cc. water were added and the flasks plugged and autoclaved at 120°C. for one and one-half hours.

The experiments which immediately follow refer to studies of the inhibition of the fermentation of maize. In each experiment flasks containing 6 grams of maize in 3 per cent concentration were acidified with varying amounts of the acids whose inhibiting concentrations were under investigation. After standing for only such time as was required for the solution of the acid, each flask was inoculated with approximately 0.5 cc. of well-shaken maize culture of the organism, about twenty-four hours old. Usually the inoculum was neutralized just before being used. The flasks were fitted with Alwood fermentation valves containing concentrated H_2SO_4 and were then weighed at room temperature and incubated at 37 to 38°. Initial molar concentrations of the added acids were calculated from data obtained by titrating accurately measured quantities of the various acids in aqueous

solution, with N/10 NaOH. Initial pH values were obtained by direct measurement on samples removed aseptically from flasks of an exactly similar duplicate series. The latter were also incubated and served to provide qualitative confirmation of the inhibiting concentrations revealed by the experimental series. At the end of the incubation period the experimental flasks were weighed at room temperature. From a consideration of the losses in weight, conclusions were drawn as to the initial inhibiting concentrations of the acids added. In tables 1 to 5, inclusive, are recorded data which pertain to the influence of thirty representative acids. An ideal experiment would have been one in which the effects of these acids were investigated simultaneously under identical conditions; this was impossible, so that one had to be content with studies of smaller groups of acids. The effects of acids grouped together in any single experiment were studied simultaneously except where otherwise stated; it will be observed that in the case of many of the acids two or more experiments are reported; the significance of the variations will be discussed later. In table 5 condensed data referring to Experiments 5 to 10 are recorded, the figures having been derived from findings of the sort detailed for the first four experiments, but which, for the sake of brevity, have been omitted in the case of the later experiments.

Experiment 1

The data relating to this experiment are summarized in table 1. If, in the case of each acid, one plots degree of fermentation against initial pH one obtains a curve with the aid of which it is possible to estimate accurately the inhibiting pH for that acid. When this is done the values recorded in table 2 are obtained. It is observed that, in the case of most of the acids, flasks having an initial reaction of pH 3.90 to 3.70 failed completely to ferment. Pyruvic acid was a notable exception, the inhibiting reaction in this case being between pH 3.51 and 3.25. Caproic acid also stood apart from the others, inhibiting the fermentation at a reaction of pH 4.4. The same general zoning of the acids can be observed if one employs, not complete inhibition of fermentation but rather 50 per cent fermentation as an index of the degree of acid influence.

TABLE 1

Experiment 1. Fermentation of 3 per cent maize as affected by added acid

ACID	CONCENTRATION OF ADDED ACID	FLASK 1			FLASK 2			FLASK 3			FLASK 4			FLASK 5			FLASK 6		
		a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
Sulphuric.........	1.98 N	0.05	5.45	2.17	0.10	5.04	2.07	0.20	4.40	1.43	0.30	3.91	0.32	0.40	3.52	−0.03	0.50	3.21	No fermentation
Hydrochloric......	2.04 N	0 05	5.54	2.30	0.10	5.14	2.21	0.20	4.38	2.02	0.30	3.82	0.21	0.40	3.42	−0.04	0.50	3.10	No fermentation
Nitric...........	2.00 N	0.05	5.55	2.19	0.10	5.12	2.09	0.20	4.39	2.17	0.30	3.91	0.19	0.40	3.40	0.00	0.50	3.09	No fermentation
Phosphoric........	3 N	0.30	4.72	2.05	0.40	4.37	1.99	0.50	4.06	0.80	0.60	3.78	0.03	0.70	3.57	0.02	0.80	3.41	No fermentation
Acetic	Pure acid	0.05	4.62	2.18	0.10	4.30	2.15	0.20	3.93	2.18	0.30	3.73	1.18	0.40	3.64	−0.04	0.50	3.58	No fermentation
Butyric..........	Pure acid	0.30	4.03	2.14	0.40	3.92	2.16	0.50	3.84	0.01	0.60	3.79	0.73	0.70	3.75	0.15	—	—	—
Caproic..........	Pure acid	0.05	4.98	2.28	0.10	4.75	2.24	0.15	4.58	2.30	0.20	4.46	0.59	0.25	4.38	0.09	0.30	4.30	No fermentation
Succinic..........	1.00 N	0.50	4.98	2.19	1.00	4.46	2.19	2.00	4.10	2.14	3.00	3.92	0.01	4.00	3.80	−0.02	5.50	3.65	No fermentation
Tartaric..........	2.00 N	0.25	4.67	2.14	0.50	3.78	1.93	0.75	3.53	1.03	1.00	3.39	0.19	1.25	3.28	0.14	1.50	3.19	No fermentation
Maleic...........	2.00 N	0.25	4.98	2.27	0.50	4.18	2.07	0.75	3.62	−0.02	1.00	3.20	−0.05	1.25	2.98	0.00	1.50	2.85	No fermentation
Crotonic..........	0.50 N	3.00	4.13	2.44	4.00	4.02	2.29	5.00	3.95	2.32	6.00	3.90	2.19	8.00	3.78	−0.02	10.00	3.66	No fermentation
Pyruvic..........	20% aqueous solution	0.10	4.72	2.29	0.20	3.93	2.29	0.30	3.51	2.32	0.40	3.25	−0.02	0.50	3.08	0.05	0.60	2.96	No fermentation
Levulinic.........	Pure acid	0.10	4.45	2.10	0.20	4.13	2.04	0.30	3.95	0.56	0.40	3.85	−0.01	0.50	3.75	0.00	0.60	3.67	No fermentation
Control flask.....	—	—	6.02	2.30															

a = cubic centimeters of acid added to substrate containing 6 grams maize in 200 cc. water.

b = initial pH after addition of acid and inoculum.

c = grams loss in weight after six days' incubation at 37 to 38°.

Experiment 2

This experiment is similar to the first, except that nitric and hydrochloric acids have been omitted, the first experiment having indicated that the three mineral acids behave similarly, as one would expect. Heptylic acid was included in experiment 2 and the range of initial concentration of all acids was considerably narrowed. Figure 1 illustrates the inhibiting relationships of the acids in terms of initial pH. Here again, can be observed the fact that the inhibiting effect of certain of the acids appears to be associated definitely with the factor of hydrogen ion concentration, the initial inhibiting concentrations of these acids falling within

TABLE 2

Experiments 1 and 2. Initial pH associated with inhibiting concentrations of various acids added to 3 per cent maize

	SUL-PHURIC	HYDRO-CHLORIC	NITRIC	PHOS-PHORIC	ACETIC	BUTY-RIC	CAPROIC	
pH	3.78	3.78	3.79	3.72	3.68	3.73	4.37	Experiment 1 (Control = pH 6.02)
	3.74	—	—	—	3.70	3.77	4.39	Experiment 2 (Control = pH 5.32)

	SUC-CINIC	TAR-TARIC	MALEIC	CRO-TONIC	PYRUVIC	LEVU-LINIC	HEPTY-LIC	
pH	3.92	3.38	3.62	3.78	3.50–3.25	3.88	—	Experiment 1
	3.97	3.56	3.67	3.77	3.18	3.77	4.65	Experiment 2

the range of pH 3.80 to 3.65, approximately. In both experiments the initial inhibiting reaction in the case of succinic acid appears to be shifted slightly to the more alkaline side of this zone. The divergence, however, is not great. Experiment 2 reveals again an apparently detached position of pyruvic acid as well as an unmistakable difference in the behavior of caproic and heptylic acids on the one hand and the remaining acids on the other.

There seems to be a legitimate conclusion to be drawn from these two experiments, namely that certain of the acids so far studied—sulphuric, nitric, hydrochloric, acetic, butyric, crotonic, levulinic, maleic, phosphoric and possibly succinic—inhibit the

acetone-butyl alcohol fermentation when present initially in such amount as to establish a hydrogen ion concentration which falls definitely within a narrow zone. In other words, in the case of these acids, one is concerned primarily with a pH effect; although other factors probably exert secondary influences, we are reasonably justified in concluding that hydrogen ion concentration takes first place among the factors associated with the inhibitory influence of any of the acids just mentioned. The so-called inhibiting zone of initial pH to which reference has been made is
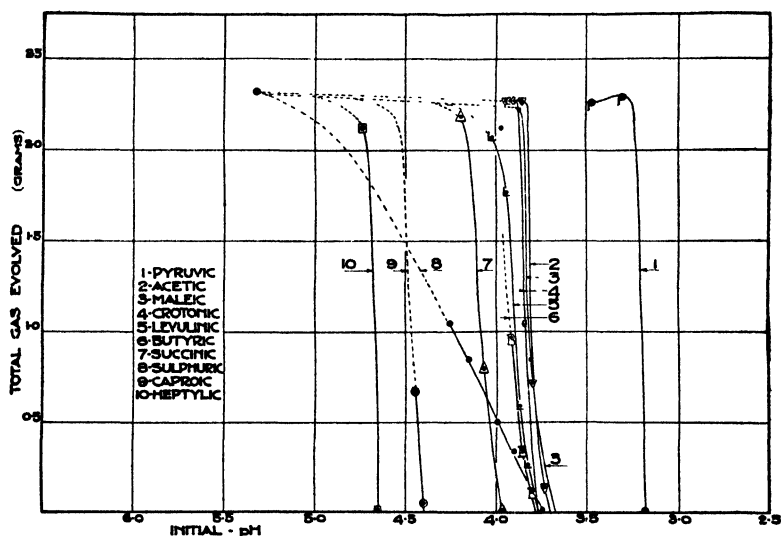
Fig. 1. Degree of Fermentation in Relation to Initial Hydrogen Ion Concentration Established by Different Acids (Experiment 2)

apparently not a fixed region. The two experiments whose results have just been recorded indicate that this is true, and many other similar experiments have yielded results which fail to establish evidence of an unchanging and invariable region of initial inhibiting hydrogen ion concentration. Summarized data derived from such experiments and recorded in table 5 make this point clear. Several factors are involved in this variability; they relate both to the nature of the substrate and to the physico-chemical behaviour of the cells introduced as inoculum. It is extremely

unlikely that throughout its life history the organism exhibits a fixed physico-chemical nature; investigations of the life cycles and the dissociation of numerous bacterial species (Löhnis, 1921), Hadley (1927), Cunningham (1931) reveal the improbability of such stability. Variations are, therefore, to be expected in the reactions of cells which, though of similar origin and age, are presumed frequently to manifest profound differences of structure and behaviour, although it is not always possible accurately to discern and measure these.

Experiment 3

In view of the suggestion in the previous experiment that certain of the lower fatty acids behave differently from other representative organic acids and the mineral acids, a more extensive study of inhibition by the lower fatty acids was made. In figure 2 data derived from such a study are presented graphically. For each acid it is observed that there is a certain critical initial reaction, acidification beyond which causes an immediate and very marked diminution in the degree of fermentation. Moreover, the inhibiting initial pH levels are obviously not the same for all the acids. Formic, acetic, propionic, butyric and isobutyric acids appear to bring about inhibition at initial pH values which fall within a narrow zone, approximately pH 3.75 to 3.65. But, with valeric and isovaleric acids, one observes a slightly greater toxicity which becomes progressively more apparent in the higher homologues, caproic, heptylic, caprylic and nonylic acids. Inhibiting concentrations of these last four acids are associated in this experiment with initial pH values of approximately 4.35, 4.75, 5.00 and 5.10 respectively, as compared with pH 3.90 for valeric acid and the zone previously mentioned for the lower homologues. The experiment was repeated several times and in every case the same general pH relationships were observed for these acids. All of the pH values which have just been recorded refer to conditions which bring about complete inhibition. The homologous acids bear similar relations to each other when 50 or 75 per cent fermentation is arbitrarily chosen as the basis of comparison. Experiment 3 is strictly comparable with Experiment I since the initial

reactions of the control flasks in the experiments were practically identical, pH 6.02 in experiment 1 and pH 6.07 in experiment 3. It is observed that the initial inhibiting reactions are very nearly identical in both experiments in the case of the acids which are common to both, namely acetic, butyric and caproic.

It is evident, however, that some influence other than hydrogen-ion concentration is at work in the case of the higher members of the series. If, in experiment 3, the toxic properties of the fatty
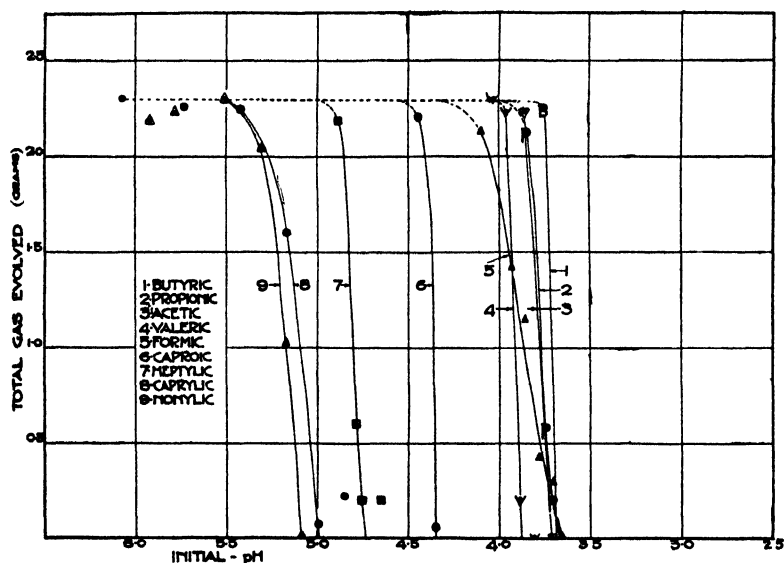

FIG. 2. DEGREE OF FERMENTATION IN RELATION TO INITIAL HYDROGEN ION CONCENTRATION ESTABLISHED BY THE LOWER FATTY ACIDS (EXPERIMENT 3)

acids are examined from the point of view of initial molar concentration rather than CH_+ the inhibiting concentrations detailed in table 3 are observed. We have here a suggestion of an interesting relationship among the intermediate acids, butyric, valeric, caproic, heptylic and caprylic, but the range of variation of acid concentration in this experiment is somewhat too great to furnish an adequate basis on which to interpret the action of the acids.

Experiment 4

The experiment was therefore repeated with the same acids, omitting formic and isobutyric, but with the range of concentration of each considerably narrowed in order to define more accurately the inhibiting molar concentrations. The data per-

TABLE 3

Experiment 3. Concentrations of fatty acids causing inhibition of the fermentation of 3 per cent maize

ACID	MOLAR CONCENTRATIONS $\times 10^{-2}$	pH
Formic........	Between 0.40 and 0.45	3.66
Acetic....,.........	Between 1.72 and 2.58	3.71
Propionic.	Between 3.01 and 3.35	3.67
Butyric........	Between 2.16 and 2.70	3.68
Isobutyric...............................	Between 2.16 and 2.70	3.76
Valeric	Between 1.47 and 1.68	3.88
Isovaleric...............................	Between 1.68 and 1.89	3.85
Caproic.................................	Between 0.56 and 0.68	4.35
Isocaproic..............................	Between 0.56 and 0.68	4.34
Heptylic................................	Between 0.27 and 0.34	4.73
Caprylic..........................	Between 0.17 and 0.23	5.00
Nonylic.............	Between 0.22 and 0.27	5.10

TABLE 4

Experiment 4. Concentrations of fatty acids causing inhibition of the fermentation of 3 per cent maize at 37 to 38°

	ACETIC	PROPIONIC	BUTYRIC	VALERIC	ISO-VALERIC
Molar concentration $\times 10^{-2}$........	3.32	3.20	2.80	1.42	1.45

	CAPROIC	ISO-CAPROIC	HEPTYLIC	CAPRYLIC	NONYLIC
Molar concentration $\times 10^{-2}$........	0.80	0.82	0.35	0.22	0.22

taining to this experiment are plotted in figure 3, the charts correlating initial concentration and degree of fermentation. The inhibiting concentrations derived from this figure are recorded in table 4; their significance is discussed later.

The inhibiting molar concentrations of the various acids are

not absolutely invariable from experiment to experiment although the acids occupy the same relative positions with respect to their toxicities. The greatest variations are exhibited by the three lowest members of the series, acetic, propionic and butyric, omitting, for the moment, formic acid which, in many of its properties, seems to stand apart from the others. The evidence suggests that these three acids to a greater extent than the higher homologues owe their toxic influence to purely hydrogen-ion effects;
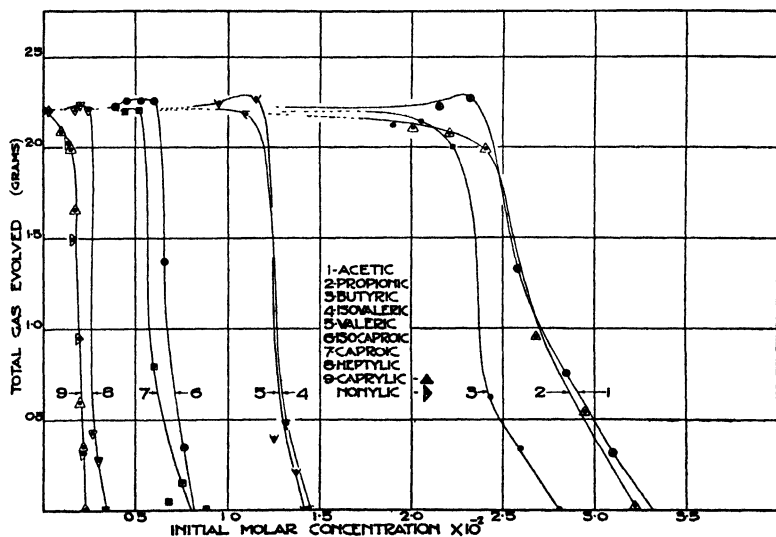
FIG. 3. DEGREE OF FERMENTATION IN RELATION TO THE INITIAL MOLAR CONCENTRATION OF THE LOWER FATTY ACIDS (EXPERIMENT 4)

any factors which cause variation in the dissociation of the acid naturally affect the total amount of acid necessary to bring the culture medium to a given level of CH_+. And, since the dissociation constants of these acids are very low—of the order of magnitude 1.5×10^{-5}—small variations of initial CH_+ involve relatively large differences of total acid. We find therefore upon examining the results of other experiments similar to experiments 3 and 4 that, particularly in the case of acetic, propionic and butyric acids, inhibiting molar concentrations vary quite appreciably. The variations in the case of the higher acids are not

TABLE 5

Summary of further experiments on the determination of the initial concentrations of acids which inhibit the fermentation at 37 to 38°C.

EXPERIMENT NUMBER	ACID	MOLAR CONCENTRATION $\times 10^{-2}$	pH	SUBSTRATE (MAIZE)	INITIAL pH OF CONTROL
				per cent	
5	Acetic	6.24	3.80	3	6.78
	Propionic	5.17	4.00		
	Butyric	5.20	3.92		
	Valeric	1.60	4.54		
	Caproic	0.85	5.30		
6	Valeric	Between 1.47 and 1.68	Between 4.13 and 4.04	3	6.00
	Isovaleric	Between 1.26 and 1.47	Between 4.17 and 4.07		
	Caproic	Between 0.68 and 0.80	Between 4.60 and 4.51		
	Heptylic	0.28	4 97		
7	Acetic	6.0	3.55	3	6.10
	Propionic	Between 5.3 and 6.0	3.63		
	Butyric	Between 5.4 and 5.9	3.65		
	Valeric	Between 1.5 and 1.9	3.95		
	Isovaleric	Between 1.5 and 2.0	3.96		
	Caproic	Between 0.60 and 0.80	4.62		
	Isocaproic	Between 0.60 and 0.85	4.65		
	Heptylic	Between 0.25 and 0.37	4.95		
	Caprylic	Between 0.12 and 0.20	5.14		
8	Formic	0.38	3.67	3	5.36
	Acetic	3.44	3.47		
	Propionic	3.45	3.50		
	Butyric	3.80	3.47		
	Isobutyric	3.85	3.48		
	Caprylic	0.21	4.52		
	Nonylic	0.14	4.80		
	Glycollic	0.45	3.62		
	Lactic	1.25	3.25		
	Glyceric	0.90	3.14		
	β-hydroxy-butyric	1.90	3.60		
9	Tartaric	1.0	3.43	3	6.69
	Malonic	1.3	3.87		
	Maleic	0.65	4.04		
	Sulphuric	—	3.95		

TABLE 5—*Concluded*

EXPERIMENT NUMBER	ACID	MOLAR CONCENTRATION $\times 10^{-2}$	pH	SUBSTRATE (MAIZE)	INITIAL pH OF CONTROL
				per cent	
10	Acetic	5.2	3 40		
	Monochloracetic	Between 0.005 and 0.01	Between 5.55 and 5.45		
	Dichloracetic	About 0.025	About 5.40	3	5.73
	Trichloracetic	Between 0.008 and 0.018	Between 5.50 and 5.40		

nearly so pronounced, suggesting again that their effect is bound up more intimately with the undissociated molecule.   In table 5 are summarized data derived from other experiments illustrating the variations of which we have spoken.

DISCUSSION

The data summarized in tables 1 to 5 indicate at once a lack of complete uniformity in the results of different experiments. There seems to be no justification for the assertion that any given concentration of hydrogen ions is at all times completely inhibitive, or capable of causing any given degree of inhibition.   We have observed for example that with pyruvic acid, under certain conditions, a concentration equivalent to pH 3.2 was necessary to effect complete inhibition, whereas with nonylic acid under similar conditions a much lower concentration of hydrogen ions was associated with an equal inhibitory effect, namely pH 5.1.   Similarly, whereas in one instance acetic acid at a level of pH 3.4 prevented the fermentation, monochloracetic acid was so much more toxic that a similar result was brought about by a concentration corresponding to a reaction of pH 5.4.   We must conclude therefore that factors other than the hydrogen ion, introduced with certain acids, exert profound influences on the physiological activity of the organism.   On the other hand, in the case of many acids the evidence points to a preponderating hydrogen ion effect.   This

seems to be true for hydrochloric, sulphuric, nitric, phosphoric and many representative organic acids including the lower fatty acids, formic, acetic, propionic and possibly butyric and isobutyric as well as hydroxy derivatives of acetic propionic and butyric acids, crotonic, levulinic, succinic, tartaric, malonic and maleic acids. Among these acids there is not always complete uniformity of toxicity expressed in terms of hydrogen ion concentration; but the evidence which we have accumulated suggests that, if it were possible completely to control all other variable factors, hydrogen ion concentration would be revealed as the predominant inhibiting factor associated with the effects of these acids. Evidence of the type provided by experiments 1, 2, 8 and 9 lends support to this conclusion.

Reference has already been made to the apparent relatively high tolerance of the organism for pyruvic acid. On one occasion, not hitherto cited in this paper, a maize flask fermented to completion, whose initial reaction, established by the addition of pyruvic acid, was pH 2.95, quite an abnormal level. This phenomenon of high tolerance has been observed also in isolated instances with other acids, such as lactic and glyceric in experiment 8. The fact that these three acids are 3-carbon atom compounds bearing close relationships one to another and to intermediate compounds which have been postulated for inclusion in the metabolic progression of the organism is of possible significance in this connection. Further investigation of this point is, however, desirable before attempting to formulate a definite conclusion.

The introduction of chlorine into the acetic acid molecule renders this acid very much more toxic. The dissociation constants of the three chloracetic acids are much higher than the acetic acid constant, and very much smaller amounts of these acids are necessary to cause complete inhibition of the fermentation, as illustrated in table 5, experiment 10. However, from a consideration of the inhibiting concentrations it is evident that some factor other than hydrogen ion concentration is here involved. It would seem unlikely that speed of penetration is a factor of primary importance since the induction period through which the organisms normally retain their viability should provide more than sufficient time for

the penetrating acid to establish an equilibrium between intracellular and extracellular concentrations. Rosenblatt and Rozenband (1909) observed a similarly greater toxic effect of the chloracetic acids as compared with acetic in the case of alcoholic fermentation by yeast. Recently Luundsgaard (1930) has observed that monoiodoacetic acid in concentration of 0.001 M inhibits the fermentation of sugar by yeast when the latter is present in relatively high concentration. It is evident that halogen substitution derivatives of acetic acid have a marked influence on the fermenting mechanism of microörganisms.

The use of buffer salts to establish different levels of hydrogen ion concentration has been deliberately avoided for the reason that their addition to bacterial cultures has often, undoubtedly, a more profound effect than that involved in the mere regulation of the reaction.

On the alkaline side of neutrality, as on the acid side, maize mash, beer-wort and glucose-peptone are fermented by the organism over a wide pH range. The establishment of unchanging pH levels by the addition of NaOH to carbohydrate substrates is impossible and therefore, where buffers are not employed, the alkaline tolerance of the organism and its fermenting mechanism can be only approximately defined. Experiments have shown that the organism grows and functions relatively normally in a medium whose initial reaction is pH 11.0. The processes of fermentation of course immediately produce a more acid reaction.

In their toxic effects on the fermentation the lower fatty acids exhibit certain interesting relationships. Among these acids it is observed that there is a noteworthy lack of general uniformity of inhibiting power at equivalent concentrations. It has already been pointed out that our evidence indicates that formic, acetic, propionic and possibly butyric and isobutyric acids owe their toxicity primarily to the influence of a certain "critical" concentration of hydrogen ions. With the exception of formic acid these acids have very low dissociation constants and, therefore, one might be led to suspect that their toxic effects must be dependent largely upon the concentration of undissociated acid rather than of hydrogen ions. But the fact that the inhibiting concentrations

of these acids fall within the pH zone which includes a considerable number of other representative organic and inorganic acids indicates that there is probably no essential difference in the cause of the inhibition in all these cases: a limiting hydrogen ion concentration appears to be the important factor. Further experiments on the effects of the lower fatty acids in the presence of their sodium salts may assist in establishing the validity of this statement in so far as it refers to these acids. It must be remembered that the acids which cause inhibition at equivalent CH_+ levels exhibit very marked differences in the speeds with which they penetrate into living cells. But speed of penetration is probably not an important limiting factor under conditions such as those which prevail in our experiments. When one examines the effects of acids higher in the series than butyric, one finds that CH_+ as a controlling factor must take a secondary position. In experiment 3 (fig. 2) for example it is observed that butyric, valeric, caproic, heptylic, caprylic and nonylic acids inhibit the fermentation at initial pH levels of 3.68, 3.88, 4.35, 4.73, 5.00 and 5.10 respectively: it is impossible to escape the conclusion that CH_+ has here only secondary significance and that the real cause of the paralysis of the normal physiological functioning of the organism is associated with some other factors or phenomena.

From the point of view of molar concentration it is observed that acetic, propionic, butyric and isobutyric acids are equally toxic at approximately equivalent concentrations. This uniformity is probably merely a reflection of the close resemblance of the dissociation constants of these acids; the variations in the inhibiting molar concentrations of these acids from experiment to experiment are due, no doubt, to factors which influence the ionization of the acid, to the general nature of the culture medium and to variation in the vitality of the inoculum. The higher homologues, from valeric to nonylic, are quite definitely tolerated to a much smaller extent than are the lower acids and there is evidence of a regularly decreasing tolerance as we ascend the series. Though our experiments do not permit us to state specifically the concentrations of the higher acids which at all times effect inhibition of the fermentation, nevertheless the variation, from experi-

ment to experiment, in these effective concentrations is relatively small. Data pertaining to butyric, valeric, caproic, heptylic, caprylic and nonylic acids point to the conclusion that to obtain the same degree of inhibition the required amount of each successive higher homologue must be about one-half to one-third of the concentration of the previous lower homologue (cf. tables 3, 4, 5). Nonylic acid and, to a smaller extent, caprylic acid display irregularities which can be explained on the basis of their very low solubility in the culture medium at 38° and which therefore do not seriously affect conclusions which it is possible to deduce from the behaviour of the other acids.

TABLE 6

Relative capillary values of the lower fatty acids (Traube "rule")

	FORMIC	ACETIC	PROPIONIC	BUTYRIC	VALERIC
Capillary value (c).	1.38	0.352	0.112	0.051	0.0146

	ISO-VALERIC	CAPROIC	HEPTYLIC	CAPRYLIC	NONYLIC
Capillary value (c)...	0.0158	0.0043	0.0018	0.00045	0.00014

"c" is a constant for each acid, representing the concentration which causes a lowering of about 14 per cent in the surface tension of water. The above data are reproduced from Freundlich "Colloid and Capillary Chemistry," 1926, p. 65.

This general relationship at once suggests Traube's rule as it applies to the lower fatty acids in aqueous solution. In effect, this rule states "that the surface activity increases strongly and regularly as we ascend the series. Thus, in order to get the same lowering of surface tension of water we need of each successive higher homologue about one third of the concentration of the previous member which is smaller by one CH_2 group" (Freundlich p. 64). These relations are illustrated in table 6. Furthermore, our experiments indicate also that isomeric homologues are approximately equally toxic at equal concentrations. As Freundlich points out, isomeric substances have almost equal capillary values and therefore lower the surface tension of water about equally. Related to this property of capillary activity is that property of

many substances which affects their adsorbability by materials presenting a large surface, such as blood-charcoal. It is generally true that the adsorption of organic substances from solution in water and other polar liquids by non-polar solids increases regularly as we ascend an homologous series. The application of these considerations, however, to systems involving bacterial suspensions may be misleading. Bacterial cells in suspension undoubtedly present large surfaces suitable for the adsorption of accessible substances but it is doubtful if we are yet in a position to conclude that a relationship which has been shown to exist for the adsorption of organic substances by systems represented by blood-charcoal necessarily always holds equally well when the adsorbing surfaces are those of bacterial cells. The very properties which we associate with the living cell imply a comparative instability of the molecular structure with consequent variation in the physico-chemical nature and behaviour of the organism. That such variation might manifest itself in a changing capacity for the adsorption of the sort of compounds we have been considering would seem to be a possibility. Therefore, one hesitates to postulate a strict parallelism between the data derived from these inhibition studies and the data of Freundlich and others which refer to much more completely understood physical systems. But is not this apparent adsorbing faculty of the cell merely related to the possible lipoid nature of the cell membrane which adsorbs the capillary active substance by simple solution? Warburg has demonstrated (Michaelis, 1925, p. 62) that erythrocytes freed of all lipoid substances are able to adsorb capillary active substances in a manner identical with that shown by inanimate charcoal models. It is probable that bacterial cells, if it were possible to free them of lipoids, would behave similarly. Conclusions as to the significance of lipoid solubility in this connection are governed by definitions of adsorption. The adsorbed fatty acid is attracted by both the aqueous phase and the solid (probably lipoid) phase, and according to the generally accepted view it constitutes a monomolecular layer on the cell surfaces, with the molecule so oriented that the non-polar end of the chain is attached to the surface of the cell whilst the polar -COOH group extends into

the aqueous phase. Recent studies of Trillat (1929) demonstrate very clearly this type of arrangement. Using a method involving spectrographic examination of x-rays diffracted by fatty acids on the surface of mercury, he was able to demonstrate that the acid molecule is so oriented that its -COOH group is attached to the mercury whilst the carbon chain extends into the gaseous phase. With increasing number of C atoms there was observed for each additional atom a regular increase in the lattice-spacing. This type of experiment has led to the view that similar orientations prevail when fatty acids in aqueous solution are adsorbed by living cells. The phenomenon in such cases appears to represent a state of equilibrium involving attractions of the polar and non-polar portions of the molecule by two different phases, a conception which implies a certain degree of solution in each phase. Distinction between the adsorption by bacterial cells of fatty acids in aqueous solution and the solubility of the acids in the two phases would seem merely to emphasize differences in the equilibrium position of which we have spoken. Whatever may be one's precise definition of adsorption the phenomenon in every case involves concentration at an interface, and it is this accumulation of adsorbed substance which seems to exert some profound influence on the metabolic processes of the cell. Merely to state, however, that the inhibitory effects of caproic acid are due to the adsorption of this acid at the cell surfaces does not explain the mechanism of the inhibitory action. One can only speculate as to what constitutes the essential physiological effect of this concentrated layer of adsorbed substance, resulting in the failure of the organism to function normally.

Adsorption of certain capillary active substances, such as the saturated paraffins, by bacteria is frequently possible without any noticeable effect on metabolism: the influence of adsorbed fatty acids is therefore due to something more than the mere physical presence of a foreign substance. Recent investigations and speculations of Quastel and his collaborators (1926, 1927) are interesting in this connection. They believe that the enzymic activities of microörganisms are due to the presence, on the cell surfaces, of electric fields of varying intensity, some of which are strong

enough to bring about activation of certain substrate molecules
and others of which are not so powerful. In other words cell
surfaces can be compared with those of other heterogeneous
catalysts which exhibit regions of graded activity. It is con-
ceivable that adsorbed fatty acids, through the influence of their
polar groups, may, by causing electronic disturbances, bring
about changes in the ability of certain of the active centres to
accomplish the function for which they are normally responsible,
and that both the degree of adsorption and the distance of the polar
group of the acid from the cell surface may govern the magnitude
of the effect. Furthermore, the presence of the adsorbed layer
may affect the accessibility of the substrate molecules to the active
areas which are normally concerned with their degradation. One
must not lose sight of the possibility that at least part of the inhibi-
tory effect of the capillary active acids is due to their adsorption
by the colloidal substrate particles, rendering the latter immune to
enzymic attack. Recent studies of Borissovsky and Wwedensky
(1930) on the inhibition of the action of salivary diastase on starch
by butyric, valeric and heptylic acids suggest such an explanation.

Somewhat similar in a general way to the results which we have
obtained with the fatty acids are the results, of experiments on
the activation of starfish eggs by acids recorded by Lillie (1926).
Lillie was concerned with phenomena of activation rather than of
inhibition, but it is not unlikely that the essential cause of the two
effects can be ascribed to the same general influences. Lillie
found that, among the fatty acids, acetic, propionic and butyric
were closely similar in their activating powers; that is, the molar
concentrations at which these acids produced complete activation
in the same time and at the same temperature were almost identical,
namely 0.25, 0.24 and 0.22×10^{-2} M respectively (ten minutes at
$20°$). Valeric and caproic acids in concentrations of 0.18 and 0.14
$\times 10^{-2}$ M accomplished a similar effect in the same time. This
definite increase in activating power on passing from butyric to
valeric Lillie interprets as indicating "that adsorption as a factor
in the action of the acid first becomes relatively important with
valeric acid" (p. 345).

As to the site and mechanism of activation Lillie believes the

process to be an effect of the undissociated molecules in the external solution, a conclusion based on his observations that acetate ions and hydrogen ions acting by themselves in concentrations much higher than those of the solutions used had no activating effect. Accordingly Lillie believes that the undissociated molecules penetrate into the cell interior where they are partially dissociated, the rate of activation being determined by the CH_+ at the site of the activation reaction within the cell. This conception is based, to some extent, on the belief that the undissociated molecules penetrate much more rapidly than the ions and that therefore the latter, entering as such from the exterior, have a relatively negligible activating effect. This would appear to be a logical explanation of the observed results in experiments where *rates* of action are studied; in studies such as our own, however, where speed of penetration can be said to be relatively unimportant as a controlling factor and where one is concerned with effects related to acid concentrations which are presumed to have reached conditions of equilibrium between the interior and exterior of the cells, it would seem that a somewhat different interpretation is desirable.

Leaving out of consideration for the moment the fatty acids from valeric to nonylic which, it appears, must be considered apart from other acids, it has been shown that a wide range of organic and inorganic acids brings about complete inhibition at concentrations corresponding to practically equivalent CH_+ values. That this should be the case for such markedly different acids as formic, acetic, propionic and butyric on the one hand and the mineral acids on the other, is significant. There are at least three possible explanations of the general mechanism of inhibition by acids: (1) it is the result of the influence of hydrogen ions at the outer surface of the cell, (2) it is dependent upon the CH_+ within the cell, (3) it is brought about in some manner by undissociated acid which enters the cell or which possibly exerts its effect without passing into the interior.

In aqueous solution sulphuric acid is approximately 96 to 98 per cent ionized in the dilutions in which we have used it; therefore its effect is almost entirely due to an inhibiting concentration of its

ions either outside or inside the cell. It is extremely unlikely that the effect can be attributed to the anion; therefore inhibition of normal cell activity is due to a critical CH_+ either inside or outside the cell. If outside, difficulty arises in attempting to explain effects which, cytologically, are presumed to have their origin in the interior of the cell. If inside, then in the case of completely ionized acids we must conclude that hydrogen ions penetrate into the interior. There has been in the past some expression of doubt as to the possibility of the penetration of such ions into living cells. Recently also, for example, it has been shown by Chase and Glaser (1930) that valeric and carbonic acids differ quite distinctly from sulphuric and hydrochloric acids in their ability to affect the forward movement of paramecia. The speed of movement of these organisms in media adjusted to various pH values by the addition of HCl or H_2SO_4 was identical, even after four hours, with the speed observed in the medium at pH 7.0. With valeric and carbonic acids which are known to penetrate quickly, the speed of movement after three or four hours was proportional to the CH_+ of the external medium. These results were regarded by Chase and Glaser as evidence that, within physiological limits, valeric acid brings about an increase in the CH_+ in some parts of the interior whilst H_2SO_4 and HCl are unable to do this. On the other hand, Pantin (1923) using a related organism, the amoeba, studied the velocity of pseudopodial movement in the presence of varying concentrations of hydrochloric, acetic, butyric, lactic, sulphuric and oxalic acids; the same velocity: pH curve was obtained for all of these acids, and therefore it was concluded that inhibition of amoeboid movement depends on the hydrogen ion concentration. That H_+ ions are able to penetrate into much more highly organized cells than those of the protozoa is apparent from the simple fact that sulphuric and hydrochloric acids taste sour. As to the mechanism of the penetration of H_+ ions Taylor (1928) suggests an explanation according to which the entry of a H_+ ion, by adsorption or otherwise, into the cell membrane develops an electric charge which attracts the anion of the acid into the membrane. As Taylor points out, this method of penetration is very similar to the passage of undissociated molecules since such a pair of ions is neutral.

CONCLUSION

Since acetic and sulphuric acids inhibit at the same level of external pH, and in view of the very low dissociation constant of the former as compared with that of the latter, and, furthermore, in consideration of the probable equilibrium between internal and external concentrations of undissociated acetic acid, it is not unlikely that the mechanism of inhibition involves an effect of a concentration of H-ions in the interior which closely approximates that observed in the external medium under inhibiting conditions.

In conclusion, therefore, it can be said that, except in the case of certain acids, inhibition of the acetone-butyl alcohol fermenta-

TABLE 7

Physiological effectiveness of the lower fatty acids

Loeb (1909); membrane formation, sea-urchin eggs; nonylic > caprylic > butyric > propionic > acetic > formic

Crozier (1918); sensory activation of earthworm; caprylic > caproic > formic > valeric > butyric > propionic > acetic

Lillie (1926); activation of unfertilized starfish eggs; formic > caproic > valeric > butyric $\gtreqless$ propionic = acetic

Bodansky (1928); hemolysis of red blood cells; capric > nonylic > caprylic > heptylic > caproic > isocaproic > valeric > isovaleric > isobutyric = butyric > propionic > acetic

Inhibition of the acetone-butyl alcohol fermentation; nonylic $\geqq$ caprylic > heptylic > formic > isocaproic = caproic > valeric = isovaleric > isobutyric = butyric $\gtreqless$ propionic = acetic

tion by the acids investigated is associated with a "critical" C_{H_+} in the cell interior. The precise relation between the internal and external C_{H_+} corresponding to the inhibitory concentration of each acid is controlled by a variety of factors, but general considerations indicate that the "critical" internal C_{H_+} is not greatly different, except in the case of acids with specifically toxic groups and the capillary active fatty acids, from the external value which is associated with the inhibitory effect.

When the results of our study of the effects of the lower fatty acids on the fermentation are compared with results obtained by other workers in related fields, as summarized in table 7, it is observed that the acids arrange themselves in practically the same

order in all cases: the acids higher in the series are correspondingly more effective physiologically. This order, as Crozier's work demonstrates, is essentially the order of the speed of penetration of the acids into living cells, and it is to the differences in speed of penetration that Loeb (1913) and Bodansky (1928), for example, attribute the differences of effect which they observed. But it is true, also, that the lower fatty acids are adsorbed by non-polar solids in precisely the same order as that of their relative penetrabilities. And, since speed of penetration is probably not a limiting factor in our studies, we must conclude that differences in the effective inhibiting concentrations of these acids are related primarily to the degree to which they are adsorbed by the living cell. This involves the assumption that bacterial surfaces, as adsorbents, behave in a measure like those of charcoal: reference has already been made to the possible difficulties involved in such an assumption. Küster and Bojakowsky (1912), demonstrated that the partition of phenol between anthrax spores and water followed the general adsorption isotherm of Freundlich. Whether bacteria in general behave in a similar manner is not known, but our experiments suggest that the cells of *Clostridium acetobutylicum* react toward the capillary active fatty acids in a manner which is in approximate accord with the Traube rule.

SUMMARY

1. A study has been made of the inhibition of the fermentation of maize mash, under the influence of *Clostridium acetobutylicum* (Weizmann), as effected by 30 representative inorganic and organic acids.

2. With several acids, complete inhibition was effected in those flasks whose initial reaction fell within a narrow zone the limits of which varied from experiment to experiment but which, approximately, extended from pH 3.90 to 3.65. The following acids are included in this group: hydrochloric, nitric, sulphuric, orthophosphoric, succinic, malonic, maleic, levulinic, crotonic, glycollic, β-hydroxybutyric, formic, acetic, propionic, butyric and isobutyric.

3. The toxic effects of these acids are probably associated with

a "critical" CH_+ in the cell interior, closely approximating the observed extra-cellular hydrogen ion concentration associated with the inhibitory effect.

4. The three chloracetic acids are much more toxic than acetic acid. Their effect is not one of CH_+ but is probably due to the specific influence of the chlorine atom.

5. Hydroxy derivatives of the lower fatty acids are not more toxic than the normal acids at equivalent CH_+ levels. The evidence suggests that, in the case of the 3-carbon acids, the reverse may be true.

6. Pyruvic, lactic and glyceric acids were tolerated by the organism at CH_+ levels higher than for any other acids.

7. In the lower fatty acid series, formic, acetic, propionic, butyric and isobutyric acids inhibited the fermentation at nearly equivalent CH_+ levels, but with each successive higher homologue the inhibiting CH_+ was appreciably lower: e.g., pH values of 3.65–3.75 for the first five members including isobutyric, and pH values of 3.90, 4.35, 4.75, 5.00 and 5.10, respectively, for valeric, caproic, heptylic, capyrlic and nonylic acids.

8. On the basis of molar concentration, the order of the inhibiting effectiveness of the fatty acids is as follows: nonylic $\geqq$ caprylic > heptylic > formic > caproic = isocaproic > valeric = isovaleric > isobutyric = butyric $\gtreqless$ propionic = acetic.

9. Capillary activity has relatively little effect in the case of formic, acetic, propionic and butyric acids, but with the higher homologues its influence is very marked. The inhibiting molar concentrations of the higher homologues suggest an approximate agreement with the Traube rule as it applies to the fatty acids in aqueous solution. Adsorption of the capillary active acids is probably the chief reason for the regularly increasing toxicity of these homologues.

10. The manner in which the adsorbed acids affect the physiological behaviour of the organism is discussed.

REFERENCES

BEIJERINCK, M. W. 1893 Ver. Kon. Akad. v. Wetenschappen te Amsterdam, (Section 2), 1, 1.
BERRIDGE, E. M. 1924 Ann. Appl. Biol., 11, 73.

BODANSKY, M. 1928 Jour. Biol. Chem., **79**, 241.
BORISSOVSKY, V., AND WWEDENSKY, N. 1930 Biochem. Zeits., **219, 72.**
CHASE, A. M., AND GLASER, O. 1930 Jour. Gen. Physiol., **13**, 627.
COLLETT, M. E. 1919 Jour. Exp. Zool., **29**, 443.
CROZIER, W. J. 1916 Jour. Biol. Chem., **24**, 255.
CROZIER, W. J. 1917-18 Amer. Jour. Physiol., **45**, 323.
CUNNINGHAM, A. 1931 Centr. für Bakt. II, **83**, 22.
EGGERTH, A. H. 1927 Jour. Gen. Physiol., **10**, 147.
EVANS, A. C. 1922 Jour. Immunol., **7**, 271.
FALK, I. S., AND HARRISON, R. W. 1926 Jour. Bact., **12**, 97.
FALK, I. S., AND WINSLOW, C.-E. A. 1926 Jour. Bact., **11**, 1.
FOSTER, L. F. 1921 Jour. Bact., **6**, 161.
FRED, E. B., PETERSON, W. H., AND MULVANIA, M. 1926 Jour. Bact., **11**, 323.
FREUNDLICH, H. 1926 Colloid and Capillary Chemistry, translated by H. S. Hatfield, London.
HADLEY, P. B. 1927 Jour. Inf. Dis., **40**, 1.
HALL, I. W., AND FRASER, A. D. 1922 Jour. Path. and Bact., **25**, 19.
HARVEY, E. N. 1915 Carnegie Inst. Pub. Number 211, p. 143.
HENDERSON, V. E. 1930 Physiol. Rev., **10**, 171.
JACOBS, M. H. 1924 "General Cytology," ed. by E. V. Cowdry, Chicago, p. 99.
KATAGIRI, H. 1926 Biochem. Jour., **20**, 427.
KITASATO, S. 1888 Zeits. für Hyg. u. Infektionskrank., **3**, 404.
KÜSTER AND BOJAKOWSKY 1912 Disinfektion, **5**, 193. (Quoted by Rideal.)
LILLIE, R. S. 1923 Protoplasmic Action and Nervous Action. Chicago.
LILLIE, R. S. 1926 Jour. Gen. Physiol., **8**, 339.
LILLIE, R. S. 1927 Jour. Gen. Physiol., **10**, 203.
LÖHNIS, F. 1921 Mem. Nat. Acad. Sci. (Washington) 16, 2nd mem., p. 1.
LOEB, J. 1909 Biochem. Zeits., **15**, 254.
LOEB, J. 1913 Artificial Parthenogenesis and Fertilization. Chicago.
LUUNDSGAARD, E. 1930 Biochem. Zeits., **220**, 1.
McCoy, E., FRED, E. H., PETERSON, W. H., AND HASTINGS, E. J. 1926 Jour. Inf. Dis., **39**, 457.
MICHAELIS, L. 1925 The Effects of Ions in Colloidal Systems. Baltimore.
MILLER, W. L. 1920 Jour. Phys. Chem., **24**, 562.
OVERTON, E. 1901 Studien über die Narkose. Jena.
PAINE, S. G. 1911 Proc. Roy. Soc., B, **84**, 289.
PANTIN, C. F. A. 1923 Jour. Marine Biol. Assoc., **13**, 24.
PAUL, T., AND KRÖNIG, B. 1896 Zeits. für physik. Chem., **21**, 414.
PRATT, C. 1924 Ann. Botany, **38**, 564; ibid., **38**, 599.
QUASTEL, J. H. 1926 Biochem. Jour., **20**, 166.
QUASTEL, J. H., AND WOOLDRIDGE, W. R. 1927 Biochem. Jour., **21**, 1224.
REILLY, J., HICKENBOTTOM, W. J., HENLEY, F. R., AND THAYSEN, A. C. 1920 Biochem. Jour., **14**, 229.
RIDEAL, E. K. 1923 5th Report on Colloids, Brit. Assoc. Adv. Sc., p. 31.
ROSENBLATT, M., AND ROZENBAND, M. 1909 Compt. Rend. Acad. Sci., **149**, 309.
SMITH, H. M. 1925 Amer. Jour. Physiol., **72**, 347.
SPEAKMAN, H. B. 1920 Jour. Biol. Chem., **41**, 319.

TAYLOR, N. W. 1927 Jour. Gen. Physiol., 11, 207.
TRAUBE, J. 1913 Arch. ges. Physiol., 153, 276.
TRILLAT, J. J. 1929 J. de Physique et le Radium, 10, 32.
VELLUZ, M. L. 1927 Bull. Soc. Chim. Biol., 9, 483.
WOLF, F. A., AND SHUNK, I. V. 1921 Phytopathology, 11, 244.
WYNNE, A. M. 1929 Can. Chem. and Metallurgy, 13, 172.

THE BEHAVIOUR OF ACID-FAST BACTERIA IN OIL AND WATER SYSTEMS[1]

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Mudd and Mudd (1924, 1926) made the interesting observation that non-acid-fast bacteria in the water phase of a water-oil emulsion on reaching the interface either remain in the interface or return to the water but never pass into the oil. Acid-fast bacteria on the other hand which enter the interface from the water are snapped abruptly into the oil phase. This difference in behaviour they explain as due to the presence on the surfaces of the latter organisms of mixtures of fatty substances with many non-polar groups which prevent their being wetted by water but allow them to be wetted by the non-polar liquid, the oil. These phase boundary phenomena were observed under a dark-field microscope. We have successfully repeated their experiments using tubercle bacilli and *Mycob. phlei* as acid-fast and colon bacilli and Friedländer's bacilli as non-acid-fast organisms.

A simpler though more indirect method suggested itself which gives results apparently in complete agreement with those of Mudd and at the same time permits of certain quantitative considerations impossible in direct dark-field observation.

I. DISTRIBUTION OF ACID-FAST AND NON-ACID-FAST BACTERIA IN WATER-OIL SYSTEMS

The bacteria under examination were suspended in water or Tyrode's solution in test tubes, various amounts of olive oil

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were added, usually two parts of water suspension to one part of oil, the tubes stoppered and shaken mechanically for one hour. The emulsions were then either centrifuged or allowed to stand until the two layers had separated. The concentration of the suspension of bacteria remaining in the aqueous phase was determined by comparing the opacity with that of standard suspensions of the same organism or by counting the bacteria, using Breed's procedure. Controls without bacteria were run

TABLE 1

Distribution of acid-fast and non-acid-fast organisms between aqueous and oil phases

SYSTEM	ORGANISMS USED	PER CENT OF THE ORIGINAL NUMBER LEFT IN THE WATER*
Olive oil-distilled water....	B. subtilis	100
	Es. coli	100
	B. friedländeri	100
	Pf. mallei	100
	M. beroliniensis	30
	M. leprae 65	25
	M. smegmatis	20
	M. leprae 69	20
	M. marium	15
	M. phlei	10
	M. tuberculosis, bovine	5
	M. tuberculosis, human	5
	Partially defatted tubercle bacilli	50

* The number of bacteria left in the water phase was, in these experiments, determined by comparison with a standard suspension.

in all cases in order to be certain that any cloudiness was due to the presence of suspended organisms rather than to an emulsion of oil globules in the water phase. The organisms used were *Mycob. phlei*, *Mycob. butyricus*, *Mycob. leprae*, *Mycob. smegmatis*, *Mycob. marinum*, *Mycob. beroliniensis* and two strains of *Mycob. tuberculosis*, a bovine and a human strain, as representative acid-fast species; *B. subtilis*, *Es. coli*, *Pf. mallei* and a capsulated Friedländer type as typical of non-acid-fast species.

All the acid-fast species partitioned almost completely into the

oil, so that the aqueous layer was left clear; all the non-acid-fast species remained in the water phase. The results are summarized in table 1. There seemed to be no difference between the action of the Gram-positive *B. subtilis* and the Gram-negative *Es. coli*, nor did the presence of capsules in the case of the Friedländer bacillus distinguish it in behaviour from the other non-acid-fast bacteria. All remained definitely in the water phase, evidently indicating that although chemically the surfaces of these organisms differed in many ways, all lacked the polar groups found in the coating of the acid-fast organisms. Considerable difference in the completeness of partition of the different acid-fast species into the oil was observed. This difference seemed to be correlated with the degree of acid-fastness. As indicated in table 1 the tubercle bacilli, *Mycob. phlei* and *Mycob. marinum* pass more completely, under these experimental conditions, into the oil than the *Mycob. berliniensis*, *Mycob. leprae* and *Mycob. smegmatis*. It is interesting to note too that tubercle bacilli partially defatted by Dryer's procedure pass less readily into the oil than do normal tubercle bacilli. It is evident, however, that many of the organisms which have lost most of their acid-fastness still retain a sufficient number of chemically polar groups to allow them to be wet more readily by the oil than by the water.

These results are in general agreement with Mudd's conclusions based on his dark field method of observation.

II. OTHER OILS AND FAT SOLVENTS

Several other oils were substituted for olive oil in the above procedure, boiled linseed, cocoanut, palm, whale, cod, mineral oil, melted tallow, castor fat acid, oleic acid and triolein. No significant difference could be observed between the behaviour of *Es. coli*, *B. subtilis*, *Mycob. phlei* and *Mycob. tuberculosis* in water and in these oils from the results just reported of their behaviour in water and olive oil.

Fat solvents were similar in their action to the oils. To 2 cc. amounts of heavy emulsions of *Mycob. phlei* and *Es. coli* in water, 1 cc. amounts of the following were added: carbon tetra-

chloride, ether, xylol, kerosene, benzene, petroleum, chloroform, carbon bisulphide and butyl, amyl and iso-amyl alcohols. After a short period of shaking, a frothy interface was formed in practically all cases. After more or less complete separation of the two phases, the yellow-acid-fast organisms could be seen suspended in this froth, but none could be demonstrated in the organic layer above or below. The *Es. coli* on the other hand, remained entirely in the watery layer. The partition is apparently therefore, as in the water-oil emulsions, a question of the miscibility of the waxy coat of the acid-fast organisms in the organic phase.

III. INTERFACE CONTACT

Two sets of experiments indicated the necessity of bringing the acid-fast bacteria into the water-oil interface in order to effect a migration from the water to oil. Suspensions of acid-fast organisms were made in water and placed in test tubes as used in the previous experiments and oil was then carefully added so as to overlay the water. After standing for a week most of the organisms had settled to the bottom of the tubes and very few had entered the oil phase. To a series of test tubes containing a thick suspension of *Mycob. phlei* in distilled water, varying amounts of olive oil were added, the mixtures shaken and the oil and water again allowed to separate as in the previous experiments. Where the proportion consisted of 1 cc. of water phase to 0.35 cc. or more of oil, the partition from the water to oil was approximately complete, as in the case of experiments summarized in table 1. Where the proportion of oil was less than 0.35 to 1 cc. of water phase the partition was less and decreased with a decrease in the proportionate amount of oil.

IV. INFLUENCE OF pH

In order to avoid soap formation most of the experiments were carried out with the water phase adjusted to pH 6.0; a wide range of pH, however, proved to have very little influence upon the partition. At lower pH values, 1.6 to 6.0 the aqueous layer remained clear; in more alkaline solutions the watery layer

became cloudy due to soap formation. Notwithstanding the small amount of soap formed, the acid-fast organisms partitioned more or less completely into the oil while the non-acid fast *Es. coli* and *B. subtilis* remained in the water. Acid agglutination of the *Es., coli* and *B. subtilis* occurred in solutions more acid than about pH 2.5 and the agglutinated organisms settled to the bottom of the tubes in the water phase. If acid agglutination occurred in the acid-fast organisms the clump passed into the oil.

Since this treatment of the acid-fast bacteria is unlikely to make any alteration in the chemical structure of the non-polar groups of the surface of the bacteria such results might have been anticipated.

V. THE EFFECT OF IMMUNE SERUM

Mudd and Mudd (1926) found that acid-fast bacteria, sensitized to homologous immune serum behaved more like non-acid-fast bacteria in oil and water systems. In their dark-field preparations it was observed that sensitized acid-fast bacteria were repelled from the water-oil interface like non-acid-fast organisms.

Extending the procedure used in the former experiments, series of dilutions of anti-*Mycob. phlei* rabbit serum and of normal rabbit serum were arranged in tubes to which was added a similar suspension of phlei organisms and two parts of olive oil to one part of the water suspension. The tubes were shaken for half an hour and then centrifuged for ten minutes at low speed. This resulted in the separation of a layer of oil in water emulsion from a clear watery layer. A portion of the watery layer was removed with a Pasteur pipette, the tip broken off to avoid organisms which might have adhered when passing through the oil, and the number of bacteria determined by counting according to Breed's procedure (Breed and Brew, 1916). Four smears of 1 to 100 cc. were made from each preparation. The percentage partition was calculated on the basis of the number of organisms per field before and after the treatment with the oil. A small error comes into these results from the fact that very fine droplets of oil may remain in the water phase. Dark field examination of the water phase, however, indicates that the error is not great.

The results of one such experiment are shown in table 2. It is apparent that the partition of these acid-fast bacteria from water into oil is inversely proportional to the concentration of immune serum. A greater influence might have been anticipated. It may however be noted that the immune serum in a 3 to 6 per cent concentration produced approximately the same influence on the partition of the bacilli as 20 to 100 per cent of normal serum.

TABLE 2

The effect of immune and normal rabbit serum upon the partition of acid-fast organisms, Mycob. tuberculosis, between oil and water

PER CENT OF SERUM	PER CENT IN EMULSION AFTER CENTRIFUGING	AVERAGE NUMBER OF ORGANISMS PER FIELD		PER CENT PARTITION FROM WATER INTO OIL
		Before shaking	After shaking	
Normal serum				
100	50	82	32	61
50	25	67	20	73
20	12.5	93	17	81
10	5	88	11	89
2	0	100	10	90
1	0	91	4	96
0	0	88	2	97
Immune serum				
50	25	58	45	22
25	12.5	88	71	19
12 5	10	73	41	44
6.5	5	62	23	63
3.0		64	16	75
2.0	0	90	4	93
1.0	0	65	6	94
0	0	80		92

Later observation indicated that the immune serum used in these experiments was of very low potency as shown by agglutination reactions.

These results, like the earlier findings of the Mudds (1926) previously referred to, are in conformity with the considerable work on the physical behaviour of sensitized antigens and especially with the recent observations of McCutcheon, Mudd,

Strumia and Lucké (1930).   Acid-fast bacteria sensitized with a homologous immune serum, they find, assume an isoelectric point progressively more alkaline as the concentration of serum is increased to one slightly more alkaline than that of serum euglobulin.   This, they believe is the result of the bacteria becoming coated with the serum antibody.   In this instance the action may be regarded as replacing or covering the non-polar groups of the lipoid surface of the organism with a film exhibiting polar groups immiscible with oil.

VI. EMULSIFYING AGENTS

The action of an emulsifying agent was suggested by the effect produced by normal serum on the partition of acid-fast bacteria from water into oil.   Several emulsifying substances were therefore examined.   A coarse emulsion of olive oil in water was stabilized by the addition of a small amount of gelatine.   Portions of the emulsion were then shaken with a watery suspension of acid-fast *Mycob. phlei* and other portions with non-acid-fast *B. subtilis*.   Under dark-field examination the acid-fast bacteria which came into contact with the oil globules appeared to remain in the interface rather than to pass into the oil drops whereas the non-acid-fast organisms meeting the phase boundary rebounded into the water phase.   Similar results followed the use of other emulsifying agents such as soaps and sodium and calcium oleates.

To establish these data on the quantitative basis of the previous experiments various concentrations of emulsifying agents were mixed with watery suspensions of the acid-fast *Mycob. phlei*. The suspensions were then mixed with two volumes of olive oil, shaken for half an hour and centrifuged for ten minutes.   The number of organisms remaining in the watery layer was then determined by counting, as with the experiments on immune serum.   In these experiments, as previously noted, as the efficiency of the emulsifying agent became great enough to prevent the separation the method failed.   In the higher concentrations of the emulsifying agents there are some very fine drops of oil, as shown by the dark-field and any bacteria in these drops

are likely then to be counted as in the water phase.   The method is therefore slightly loaded *against* the emulsifying agents. Notwithstanding this error the rate of partition of the bacteria from water to oil is shown in table 3 to be inversely proportional

TABLE 3

The effect of emulsifying agents upon the partition of acid-fast Mycob. phlei into oil

PER CENT OF EMULSIFYING AGENT USED	PER CENT IN EMULSION AFTER CENTRIFUGING	AVERAGE NUMBER OF ORGANISMS		PER CENT PARTITION INTO THE OIL
		Before shaking	After shaking	
a. Gelatine				
10	75	85	49	42
5	66	71	43	40
1	50	70	38	46
0 4	50	72	36	50
0.04	10	83	15	82
0 001	0	83	5	94
0	0	112	5	97
b Sodium oleate				
5	50	89	63	28
2 5	25	75	36	52
1.0	15	90	34	62
0.3	10	83	31	63
0.1	0	87	21	76
0 01	0	85	14	84
c. Normal rabbit serum				
100	50	109	36	63
50	25	92	18	80
20	12	84	9	89
10	5	85	8	90
2	0	90	5	94
0	0	98	4	96

to the concentration of gelatine, sodium oleate and, less conspicuously, normal rabbit serum.

This action might be explained according to Harkins' view of emulsions and emulsifying agents on the ground that the emulsifying agent has formed a film about the oil particles and so reduced

the free energy between the oil and water that the fatty surfaced organisms are no longer carried through the interface.   It seems equally reasonable to assume that an effective emulsifying agent may form a surface film over the fatty surface of the organisms. A stable emulsion will result, according to the Harkins' theory, if the molecules in this new interface are oriented with the dispersion medium.   One emulsifying agent might stabilize, at the same time, a water-oil emulsion and a water-*M. phlei* emulsion.

SUMMARY

1. On shaking watery suspensions of acid-fast bacteria with oil they readily pass into the oil while non-acid-fast bacteria remain in the water.   The same reaction occurs with a variety of fat solvents.

2. The partition into oil is shown quantitatively to be nearly complete for tubercle bacilli and 70 to 90 per cent for six other acid-fast species.

3. Homologous immune serum inhibits, in proportion to its concentration, the partition from water to oil.

4. Emulsifying agents such as gelatine and sodium oleate have a similar effect.

REFERENCES

BREED, R. S , AND BREW   1916   Tech. Bull. 49, New York Agric. Exp. Sta.
COULTER, C. B.   1920–21   Jour. Gen. Physiol., 3, 309.
McCUTCHEON, M , MUDD, S., STUMIA, M., AND LUCKÉ, B.   1930   Jour. Gen. Physiol., 13, 669.
MUDD, S., AND MUDD, E. B. H.   1924   Jour Exp. Med., 40, 633.
MUDD, S., AND MUDD, E. B. H.   1926   Jour. Exp. Med., 43, 127.
MUDD, S., AND MUDD, E.B. H.   1927   Jour. Exp. Med., 46, 167, 173.
MUDD, S., LUCKÉ, B., McCUTCHEON, M., AND STRUMIA, M.   1928   Colloid Symposium Monograph, 131.
NORTHROP, J. H., AND DEKRUIF, P. H.   1921–22   Jour. Gen. Physiol., 4, 655.

GUM PRODUCTION BY AZOTOBACTER CHROOCOC-CUM OF BEIJERINCK AND ITS COMPOSITION[1]

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INTRODUCTION

In a paper in the JOURNAL OF BACTERIOLOGY (September, 1929), Sanborn and Hamilton described the effect of *Azotobacter chroococcum* upon the physiological activities of cellulose destroyers. The purpose of this article is to describe the production of gum by the Azotobacter and the availability of various nutrients for this purpose, also the chemical composition of the gum produced.

The slime or gum produced by *Azotobacter chroococcum* has been discussed to some extent by Stapp (1924) and Heinze (1926). Buchanan (1909) in a discussion of gum production states: "It seems but fair to conclude from this array of evidence that there is a great possibility that bacterial slimes and gums of whatever kind are produced as a transformation or solution of the bacterial capsule." The best available description of the chemical properties of a bacterial gum is that of Buchanan (1909) who worked with *Rhizobium radicicolum*. The properties of the gum isolated by Buchanan are as follows:

In a culture fluid of 2 per cent sucrose in tap water the gum was apparently partly in solution and partly as an insoluble modification, in the form of zoogloeal masses. The gum precipitated by alcohol was in the form of a soft rubbery mass when moist and hard and brittle when dry.

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[1] Parts I and III of a thesis presented to the Faculty of Graduate Studies and Research of McGill University in September, 1928, for the degree of Master of Science.

The gum was precipitated by 65 per cent alcohol. The precipitate redissolved readily on the addition of water. It was also precipitated by ammonium sulphate and ammoniacal lead acetate. No precipitation occurred with sodium chloride, lead acetate, mercuric chloride, copper sulphate, acetic, hydrochloric, nitric, sulphuric, picric and tannic acids. Magnesium sulphate caused a precipitation only after the complete saturation of the solution with crystals and in the course of twenty-four hours.

The gum reduced Fehling's solution only after autoclaving for an hour at one atmosphere pressure with 2 per cent sulphuric acid. Millon's xantho-proteic, and biuret tests for proteins were negative. A test for combined nitrogen, by converting the gum into a cyanid and obtaining the Prussian blue reaction, was also negative.

ISOLATION OF THE AZOTOBACTER CHROOCOCCUM OF BEIJERINCK

Ten grams of soil, which was classified as loam, were added to 150 cc. of sterile Ashby's solution in a large Erlenmeyer flask. The mixture was incubated at 30°C. for two days when loopfulls of the surface film were streaked on Ashby's agar. After two days' incubation on the agar these streaks showed the characteristic colonies of Azotobacter. Three species of the genus Azotobacter were fished from the above described plates.

The species of Azotobacter isolated were identified with: (1) *Azotobacter chroococcum* of Beijerinck; (2) *Azotobacter beijerinckii* of Lipman; (3) *Azotobacter vitreum* of Löhnis and Westman.

The strain of *Azotobacter chroococcum* isolated by the author was used throughout these experiments.

GENERAL PROCEDURE

The procedure for the production of gum was the same for the various nutrients used.

The nutrient solution was prepared and sterilized in the usual manner. Each flask of 50 cc. of the media used in the various experiments was inoculated with 2 cc. of the *Azotobacter chroococcum* suspension. The suspension was standardized to a turbidity of ten according to the McFarland scale.

The inoculated flasks were incubated at 30°C. for fourteen days. The gum was removed from the nutrient solutions after the

fourteen-day incubation period by a method suggested by Buchanan (1909).

The media were filtered to remove coarse particles. The filtrates were then made alcoholic to the extent of 70 per cent of the total volume. The gum which precipitated in the alcoholic solutions was separated by filtration. The separated gum was dried to constant weight and the weights recorded for the amounts of gum produced per 100 cc. of the medium used.

The gum was purified for further use by repeated dissolving and precipitating, the former with water and the latter with alcohol. The dissolved gum solution was filtered to remove any insoluble portions before each precipitation.

To preserve the gum this method was devised: To every 100 cc. of gum solution 10 cc. of di-ethyl ether were added. The ether was allowed to evaporate when the gum was required for further experiments. The gum solution contained approximately 5 per cent gum.

AMOUNTS OF GUM PRODUCED IN DIFFFRENT MEDIA

To determine the source of energy best suited to gum production by *Azotobacter chroococcum* various media were used.

1. The first medium tried was a modification of one used by Greig-Smith (1911).

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Potassium phosphate . .  . .. .. ............ ... .....     5.0 grams
Ammonium sulphate.....................................     0.6 grams
Maltose....   .......................  .. ..............  ...   20.0 grams
Distilled water. ........ ... ... . ... .... ..... ........1000.0 cc.
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The average yield of gum per 100 cc. of the above medium was 411.428 mgm.

2. A medium of glycerinated beef broth was also used in preliminary experiments. The medium contained:

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Difco beef extract........................... .................     3.0 grams
Difco peptone....................................... ......     5.0 grams
Glycerol C. P.............................................. ..   20.0 grams
Distilled water.........................................1000.0 cc.
```

The average amount of crude gum produced in this medium per 100 cc. of liquid was 2,226.50 mgm.

3. Various sugars were used in 2 per cent solutions to deter-

mine the ability of *Azotobacter chroococcum* to use them as sources of energy for gum production. The only other nutrients available were the very minute quantities of mineral salts to be found in distilled water.

The average amounts of gum produced by the *Azotobacter chroococcum* using the various sugars as sources of nutrient in concentrations of 2 per cent were:

Xylose	760.0	Glycerol	760.0
Arabinose	765.0	Mannitol	745.0
Galactose	765.0	Rhamnose	765.0
Glucose	735.0	Salacin	765.0
Laevulose	750.0	Raffinose	800.0
Sucrose	750.0	Dextrin	1,080.0
Lactose	755.0	Inulin	1,055.0
Maltose	765.0		

TABLE 1

The effect of glucose concentration, (with a fixed nitrogen source), on the production of gum by Azotobacter chroococcum

PERCENTAGE OF GLUCOSE	WEIGHT OF GUM	PERCENTAGE OF GLUCOSE	WEIGHT OF GUM
	mgm.		*mgm.*
1	770.0	7	770.0
2	800.0	8	790.0
3	790.0	9	795.0
4	785.0	10	765.0
5	795.0	15	780.0
6	810.0	20	800.0

Figures are quoted for the amounts of gum produced in 100 cc. quantities of media.

The above weights are the yields in milligrams per 100 cc. of liquid medium.

4. A nitrogen source was added to the sugars in the next series of experiments to increase the production of the gum. Peptone was used as the nitrogen source in concentrations of 0.5 per cent. The two common sugars, glucose and sucrose, were used in increasing concentrations as indicated in tables 1 and 2.

5. An attempt was made to produce gum from *Azotobacter chroococcum* in solutions of peptone alone. It was found that peptone would not furnish energy for the production of any gum.

ANALYSIS OF THE GUM PRODUCED BY AZOTOBACTER CHROOCOCCUM

1. *Physical properties*

The gum is completely soluble in water, precipitated by alcohol (65 per cent), ether or acetone. The precipitated gum is translucent and grayish-white. When dry the gum has a smooth, glossy surface. When first precipitated, the gum is a translucent, amorphous, adhesive mass which darkens on hardening. The specific rotation of the gum is $[\alpha]_D = -30.3$, which would class it with the true gums.

TABLE 2

The effect of sucrose concentration, (with a fixed nitrogen source), on the production of gum by Azotobacter chroococcum

PERCENTAGE OF SUCROSE	WEIGHT OF GUM	PERCENTAGE OF SUCROSE	WEIGHT OF GUM
	mgm.		*mgm.*
1	890.0	7	1,235.0
2	950.0	8	1,895.0
3	1,230.0	9	960.0
4	1,285.0	10	1,305.0
5	1,165.0	15	975.0
6	1,195.0	20	920.0

Figures are quoted for the amounts of gum produced in 100 cc. quantities of media.

2. *Chemical properties*

A solution of the gum (6.60 mgm. of solid gum per cubic centimeter of water) was submitted to the following tests:

1. Molisch's test: positive.
2. Fehling's test: solution not reduced after boiling with acid.
3. Biuret test: Negative.
4. Millon's test: Negative in both hot and cold solution.
5. The gum was submitted to the following precipitation tests and with these results:

   a. Gum precipitated by ethyl alcohol, 2N lead acetate, 2N ammonium sulphate, ammoniacal lead acetate.

   b. Gum not precipitated by magnesium chloride, sodium hydroxide, sodium chloride, hydrochloric acid, sulphuric acid, ammonium hydroxide, copper sulphate, acetic acid, nitric acid.

Both concentrated and 2N acids were used.

6. Test for soluble starch—When iodine was added to the gum a yellow-green colour was produced which did not change on the addition of sulphuric acid.

7. When tested with phospho-tungstic acid for Vitamine B, no precipitate was formed Williams and Seidell (1916).

8. The Micro-Kjeldahl test for combined nitrogen showed a trace. (Koch and McMeekin, 1924).

DISCUSSION AND CONCLUSIONS

Azotobacter chroococcum of Beijerinck has been shown to produce a gum using various carbohydrates as nutrients. The more complex carbohydrates are more readily available for the production of the gum than are the mono- and di-saccharides. The presence of a protein, such as peptone, enhances gum production but has not been found necessary for this function of the organism. A solution of peptone alone does not furnish material for the production of the gum.

The gum produced by *Azotobacter chroococcum* is a carbohydrate of the higher series. Boiling with acid does not cause it to reduce Fehling's solution. It is levo-rotatory and therefore of the true gums and not the dextrins.

The trace of combined nitrogen found is probably due to remains of bacterial cells not removed in the purifying process. The results of the analysis place the gum in "Class I" of Haas and Hill (1923) and under this classification it would be termed an arabin.

REFERENCES

BUCHANAN, R. E. 1909 Centralbl. f. Bakt., etc., II, 22, 371.

WILLIAMS, ROBT. R., AND SEIDELL, ATHERTON. J. Biol. Chem., 1916, xxvi, pp. 436-437.

GREIG-SMITH 1911 Centralbl. f. Bakt., etc., II, 30, 552.

HAAS AND HILL 1923 An Introduction to the Chemistry of Plant Products. Longmans, Green and Company, London, England.

HEINZE, B. 1926 Landw. Jahrb., 64, 127.

KOCH AND McMEEKIN 1924 Jour. Amer. Chem. Soc., 46, 2066.

STAPP, C. 1924 Centralbl. f. Bakt. etc., II, 61, 276.

STUDIES ON THE ELECTRICAL CHARGE OF BACTERIOPHAGE

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A common laboratory procedure for the determination of the charge carried by a dye is to dip a strip of blotting paper in the dye solution and observe whether or not the stain passes into the negatively charged paper with the water. Methylene blue, a positively charged dye, is strongly adsorbed by the paper and therefore does not rise to any extent above the solution level, although the water will rise several centimeters. On the other hand, a negatively charged dye such as eosin will climb a considerable distance in the paper with the water.

Bedson and Bland (1929) applied this method to the determination of the electrical charge carried by virus particles and their results are quite consistent with those obtained by other workers from cataphoresis experiments.

Todd (1927) by means of cataphoresis studies, found that anti-shiga bacteriophage carries a negative charge between pH 3.6 and 7.6. As a result of similar studies Krueger, Ritter and Smith (1929) reported that several races of anti-coli 'phage which they have studied carry a negative charge from pH 9.0 to 3.4 and are positively charged below 3.4. Natarajan and Hyde (1930) likewise reported that small plaque anti-coli 'phage carries a negative charge below pH 8.3, and large plaque anti-coli 'phage a negative charge below 6.1 while both seem to be amphoteric above these values. Anti-Flexner 'phage is negative between pH 5.4 and 9.3 and anti-typhosus 'phage between pH 4.9 and 9.3.

In studies on the cataphoresis of sub-visible particles, diffusion

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and endosmotic effects are difficult to control and if not carefully checked will often lead to erroneous conclusions. This is shown in the early determinations of the nature of the charge carried by the 'phage corpuscles. Thus, V. Angerer (1923–4) reported that the lytic principle migrates to the anode while Koch(1926) using a similar method reported migration to the cathode. Therefore, the migration of the 'phage in blotting paper appeared to us to be a simpler and more accurate method.

In this work strips of blotting paper 1 cm. wide and 10 cm. long are ruled with a pencil at centimeter intervals. Care must be taken in cutting these strips to obtain a sharp edge, otherwise the rate of diffusion will be irregular along the border. The strips are then sterilized.

The blotting paper was tested by noting the behavior of dilute solutions (0.01 per cent) of methylene blue, eosin and hemoglobin. The paper, either held at one end by hand or preferably by means of a clamp, is lowered 1 cm. into the fluid to be tested. It is held there until the water has risen about 4 cm. above the surface and then removed from the liquid. This usually requires about one minute. During this time methylene blue climbs about 1 cm. and eosin 3 to 4 cm. above the solution level. The behavior of hemoglobin is presented in table 1, its presence being detected by means of the benzidine reaction.

These results show that, on the alkaline side of the isoelectric point, hemoglobin ascends the strip with the water while on the acid side it is adsorbed by the blotting paper. Near the isoelectric point the results are not as sharp. In this case we are dealing with a weakly charged particle and one much more complex in its behavior than a relatively simple dye such as methylene blue or eosin.

With most biological substances one can hardly expect a sharp end point in such a test. However, the bacteriophage can be detected in quite minute amounts and therefore is more applicable to this test than the majority of viruses.

A suspension of a given 'phage in broth was tested under aseptic conditions as follows:

The blotting paper was dipped 1 cm. into the 'phage suspension

for one minute, and then cut at 1 cm. intervals above the solution level. During this latter process the fluid climbed on the average 0.5 cm. higher in the paper. These strips were then placed in a tube containing Martin's broth, the susceptible organism added and the presence or absence of lysis noted after incubation. The centimeter square of blotting paper dipped into the liquid served as a control. The results obtained for 'phages active against

TABLE 1

CENTIMETERS ABOVE SOLUTION	pH					
	2 7	4.1	5.0	6.3	7.0	8.5
0–1	+	+	+	+	+	+
1–2	±	±	+	+	+	+
2–3	0	0	0	±	+	+
3–4	0	0	0	0	0	±

+ = hemoglobin present throughout centimeter square.
± = hemoglobin present only in part of centimeter square tested

TABLE 2
E. coli (k-13), 'phage 2

CENTI- METERS ABOVE SOLUTION	pH									
	1.6	1.9	2.25	2.50	2.90	3.10	3.50	4.5	7.5	10.0
Control	0	+	+	+	+	+	+	+	+	+
0–1	0	+	+	+	+	+	+	+	+	+
1–2	0	+	+	+	+	+	+	+	+	+
2–3	0	0	0	0	±	+	+	+	+	+
3–4	0	0	0	0	0	0	+	+	+	+

+ = complete lysis.
0 = no observable lysis.

different organisms are given in table 2, only a few of the tests being reported here. The different hydrogen ion concentrations were obtained by the addition of N/10 HCl or NaOH, the pH being determined by means of a quinhydrone electrode except for the more alkaline solutions, in which case indicators were employed.

These results indicate that the isoelectric point of the bacteriophage active against *E. coli* is probably near pH 2.9.

Table 3 indicates that the isoelectric point of anti-shiga bacteriophage is near that of the anti-coli 'phage, probably close to pH 3.1.   Other tests indicate the behavior at pH 10.0 to be due to inactivation of this particular bacteriophage by alkali.

TABLE 3

E. dysenteriae (Shiga I-10), 'phage 5

CENTI-METERS ABOVE SOLUTION	pH								
	1 75	2 00	2.40	2.75	3.10	3.61	4.40	7.30	8.5	10.0
Control	0	+	+	+	+	+	+	+	+	+
0–1	0	+	+	+	+	+	+	+	+	±
1–2	0	+	+	+	+	+	+	+	+	0
2–3	0	0	0	0	±	+	+	+	+	0
3–4	0	0	0	0	0	±	+	+	+	0

TABLE 4

S. aureus (AD-52), 'phage 15

CENTI-METERS ABOVE SOLUTION	pH								
	1.70	2.20	2.85	3 25	3.50	4 0	4.5	6.0	7 5	10.0
Control	0	0	+	+	+	+	+	+	+	+
0–1	0	0	+	+	+	+	+	+	+	+
1–2	0	0	0	0	+	+	+	+	+	+
2–3	0	0	0	0	0	+	+	+	+	+
3–4	0	0	0	0	0	0	+	+	+	+

TABLE 5

B. pyocyaneus (A-8), 'phage 75

CENTIMETERS ABOVE SOLUTION	pH								
	1.70	2.10	2.65	3.00	3.30	3.60	5.00	7.20	10.0
Control	+	+	+	+	+	+	+	+	+
0–1	+	+	+	+	+	+	+	+	+
1–2	+	+	+	+	+	+	+	+	+
2–3	0	+	+	+	+	+	+	+	+
3–4	0	0	+	+	+	+	+	+	+

Table 4 indicates that the isoelectric point of this phage is near pH 3.5, decidedly more alkaline than the anti-coli or anti-shiga 'phages.

Table 5 indicates that the isoelectric point of the anti-pyocyaneus 'phage is around pH 2.10, a value considerably more acidic than those obtained for the other 'phages tested.

Inactivation tests of these different 'phages by HCl were also carried out at the same time. Five cubic centimeters of the bacteriophage suspensions were treated with the acid for five minutes and then titrated in the usual manner. Bacteriophage active against *E. coli* was inactivated in this length of time at a pH of 1.70; *E. dysenteriae* at 1.80; *S. aureus* to some extent at 2.15 and completely at 1.90; and *B. pyocyaneus* below pH 1.50.

Therefore, it appears that the values reported for the isoelectic point of the different 'phages studied are not in error due to an inactivation by these low pH values. Apparently there is also some correlation between the isoelectric points and resistance to high hydrogen ion concentrations, the 'phages having the lowest isoelectric points being the most resistant to inactivation by HCl. This likewise suggests that the structure or composition of the different 'phages is different and may probably be closely connected with the nature of the bacterium susceptible to their action. The gradual decrease in the tendency of the 'phage to climb with the water into the paper near the isoelectric point also suggests a gradual decrease in the degree of dissociation of the molecules at the surface of the lytic complex.

SUMMARY AND CONCLUSIONS

The electrical charge carried by the bacteriophage was determined by noting the tendency of this agent to climb with water in blotting paper.

The results presented herein indicate that the isoelectric point of the anti-coli 'phage tested is around pH 2.9; of anti-shiga 'phage near pH 3.1; of anti-staphylococcus 'phage approximately at pH 3.5; and of anti-pyocyaneus 'phage below a pH of 2.1.

REFERENCES

v. ANGERER, K. 1923–4 Beiträge zum Bakteriophagenproblem. Arch. Hyg., **92**, 312.

BEDSON, S. P., AND BLAND, J. O. W. 1929 A simple method for determining the electrical charge carried by virus particles. Brit. Jour. Exp. Path., **10**, 67.

KOCH, K. 1926 Untersuchungen über Bacteriophagenkataphorese. Cent. Bakt., 1 Abt., Orig., **99, 209**.

KRUEGER, A. P., RITTER, R. C., AND SMITH, S. P. 1929 The electrical charge of bacteriophage. Jour. Exp. Med., **50, 739**.

NATARAJAN, C. V., AND HYDE, R. 1930 The behavior of certain filtrable viruses when subjected to cataphoresis. Amer. Jour. Hyg., **11, 652**.

TODD, C. 1927 Electrical behavior of the bacteriophage. Brit. Jour. Exp. Path., **8, 369**.

FIVE CASES OF INFECTION OF THE URINARY TRACT DUE TO A MEMBER OF THE GROUP OF BACILLI NAMED AFTER MORGAN

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In a bacteriological examination of feces from summer diarrhea in children H. R. Morgan in 1906 found a number of lactose negative microbes belonging to the group of Gram-negative intestinal rods, which he considered of interest in the etiology of this disease. On continued investigations in 1907 he found the same species of bacteria, and in 1909 he tried to make a grouping of these bacteria, working together with J. C. G. Ledingham. The grouping was made according to the different reactions in carbohydrate-media, and eight different types were presented. The different types were again subdivided into subtypes according to the different faculty of the microbes to produce indol and according to motility or non-motility of the microbes. Morgan and Ledingham's type-scheme is given in table 1. This table gives, however, only the main types, while the unimportant subtypes are omitted. The number of each type refers to the number of each new strain that started a new type in their material. Thus type number V means the type that started with strain no. V, type X the type including the microbes similar to the strain no. X and so on. Besides the type numbers Morgan and Ledingham also use authors' names on their types, namely "Type Flexner," "Type Gaertner," and these types should include all strains belonging to microbes that are known under those names.

Of the types of Morgan's bacilli, Type I is probably the most important. The other types of Morgan's bacilli do not appear to have been dealt with to any extent by bacteriologists, neither in textbooks or in journals.

From Denmark, Type I has been described by Øhrum and Bahr (1910) and later by Bahr and Thomsen (1912). These authors, like Morgan, found Type I in feces from summer diarrhoea in children and held this microbe responsible for the disease, although they found no distinct serological evidence for this conception in the examination of blood tests from patients, suffering from summer diarrhea. Agglutination tests always showed very low titers (1:20, seldom 1:40), if they were not completely negative, as in most cases.

From Germany a similar microbe is described by E. Seligman (1917), by P. Jungman and E. Neisser (1917) and by E. Kindborg (1918) as cultivated from patients during dysentery epidemics. These authors consider the microbe as an atypical dysentery bacillus.

TABLE 1

Modified after Morgan and Ledingham

	GLUCOSE	MANNITOL	DULCITOL	LACTOSE	SUCROSE
Type I	A + G	0	0	0	0
Type Flexner	A	A	0	0	0
Type V	A	0	0	0	0
Type Gaertner	A + G	A + G	A + G	0	0
Type X	A + G	A + G	A + G	0	A + G
Type XII	A + G	A + G	0	0	A + G
Type XIV	A + G	A + G	0	0	0
Type XV	0	0	0	0	0

From Norway, Morgan's bacillus no. I is described and thoroughly studied by Th. Thjøtta (1918, 1920). He found this microbe in seven cases of acute colitis, one case ending fatally. Contrary to the other authors Thjøtta does not find any proof of responsibility of this microbe for the disease, in spite of its being cultivated in large numbers from the feces and in spite of its disappearing after the disease. He bases his opinion upon the fact that he could not find any agglutinin production in the blood of his patients, even after a fairly long spell of sickness. Contrary to this behavior in the case of human patients, the same microbes always showed themselves to be very active agglutinin producers in the animal body during experimental study. Further, all of Thjøtta's strains

were serologically different, agglutinating only in their homologous sera, but never in heterologous ones.   Thjøtta accordingly concludes that his strains may be considered as atypical strains of *B. coli*, acting as saprophytes and growing particularly well in the fecal matter altered during the colitis (pus, blood, mucus and epithelials added to the more or less fluid feces) or perhaps mutants from the ordinary lactose splitting *B. coli* of the normal intestines.

In 1928, however, Thjøtta describes a fatal case of a septic infection starting from an infection of the gall bladder and being due to Morgan's Bacillus of type I.   In this case the microbe was found in the blood and in the contents of the gall bladder.   A high titer of agglutinins and complement-fixing antibodies were demonstrated in the blood of the patient.   In this case the relationship between microbe and disease was obvious, and this case strengthens Thjøtta in his opinion with regard to the former intestinal strains, lacking serological response in the human body, as the last case definitely show, that the microbe does call forth the production of antibodies, when it is really infective.

In the same year Anton Jervell (1928), also from Norway describes two cases of urinary infection due to Morgan's bacillus no. I.   In these two cases also agglutinin production was demonstrated in the blood of the patients.

From America G. F. Dick, G. H. Dick and J. L. Williams in 1928 describe an epidemic due to *B. Morgan*.   Several cases were complicated with mastoiditis and in two cases *B. Morgan* was found in pus from the mastoid processus.   Serological examinations were not carried out, nor was the type of the microbe definitely stated.

In 1929 *B. Morgan* no. I is described by *R. d'Aunoy* as the cause of three cases of sepsis, gastroenteritis and pyelitis.   In all cases agglutinin production was demonstrated.

L. C. Havens and C. Ridgway, also during 1929, report 13 cases of paratyphoidlike disease, in five out of which *B. Morgan* was found in blood cultures and agglutinated by blood tests from the patients.

G. M. Mackenzie and Louise N. Batt in 1930 deal with an epidemic of diarrhoea. In feces from the patients they found in some cases a dysentery bacillus, in others a strain of *B. Morgan* no. I. No agglutinin production was demonstrated in blood tests from these patients.

It is obvious, that Morgan's type I has been dealt with by various authors. The other types have been completely neglected, and perhaps they should be, because they should rather be grouped under other names. It is, however, well known to every bacteriologist, that one often finds strains of bacteria during the routine bacteriological investigation of feces, that correspond to the characters of several of the microbes described by Morgan and Ledingham. In the following pages we shall give a description of five cases of infection of the urinary tract due to a microbe falling into the type of Morgan and Ledingham, named by these authors type XII.

HISTORIES OF PATIENTS

Case 1. E. L., one-year-old girl. The disease started with painful micturition, nisus and fever. In hospital a cysto-pyelitis was diagnosed, the urine containing leucocytes, pus and albumen. On bacteriological examination of the urine a strain of *B. Morgan* was demonstrated, which agglutinated in the blood of the patient up to a titer of 1:160. Blood culture showed no growth. Under vaccine treatment the fever subsided quickly and the patient recovered. The fermentation-scheme of this strain will be seen in table 2 (strain I).

Case 2. K. M., forty-year-old man. The patient was suddenly taken ill with pains in his right renal region, frequent micturition and fever. The examination of the urine showed a strain of *B. Morgan*, the characters of which are given in table 2 (strain II). A blood test ten days after the beginning of the disease showed no agglutinins against the infecting microbe, while a blood test taken two months later gave an agglutinin titer of 1:360 against the same strain.

Case 3. E. S., forty-three-year-old woman. In July, 1930, the patient sickened with frequent and painful micturition. She became, however gradually better without the help of a physician. In August she had a

relapse, which brought her to a surgeon, while she had severe pains all over her abdomen together with painful micturition and high fever. The bacteriological investigation revealed a strain of *B. Morgan* from both kidneys, while no growth was observed from the blood. An agglutination test performed in her serum gave a positive test of 1:320. The patient was fibrile for fourteen days, was treated with an autogenous vaccine and recovered quickly.

Case 4. F. S., forty-year-old man. He was suddenly taken ill with the same symptoms as the former patients. The investigation of the

TABLE 2

BIO-CHEMICAL REACTIONS IN:	STRAIN I	STRAIN II	STRAIN III	STRAIN IV	STRAIN V
Lactose	−	−	−	−	−
Mannitol..	+	+	+	+	+
Maltose	+	+	+	+	+
Glucose...........................	+	+	+	+	+
Sucrose...........................	+	+	+	+	+
Salicin............................	−	−	−	−	−
Inulin............................	−	−	−	−	−
Galactose.........................	+	+	+	+	+
Laevulose	+	+	+	+	+
Dulcitol...........	−	−	−	−	−
Isodulcitol......	+	−	+	+	+
Xylose	+	+	+	+	+
Simmons citrate tube..............	−	−	−	−	−
Indol production..	+	+	+	+	+
Gelatin liquefaction...............	−	−	−	−	−

+ = acid and gas production.
− = no fermentation.

urine showed a strain of microbes belonging to the same group as those described previously. Its fermentative characters are given in table 2 (strain IV). The strain was agglutinated up to a titer of 1:320.

Case 5. F. B., sixty-year-old woman. The patient has suffered from cysto-pyelitis for many years. She has been treated with the ordinary urine disinfectants without a result. The bacteriological examination of the urine showed in this case a microbe of the same type as in all the previous patients, and her blood test gave a positive agglutination of the strain up to 1:640.

All the five strains consist of Gram-negative rods, showing a slight motility in young broth cultures (up to twenty-four hours old). In old cultures the motility is so slight, that it cannot always be demonstrated. All strans are strong indol producers, even after a few hours of growth. The colonies of these strains are shaped like an ordinary smooth colony of *B. coli* with a clean cut edge and raised surface. They show the same density as that of coli-colonies. All are non-lactose splitting organisms, and they are all negative towards dulcitol. They show, however, a great activity towards sucrose, which is fermented with production of acid and gas. The following chemicals are also fermented and likewise with production of acid and gas: mannitol, maltose, glucose, galactose, levulose, xylose. Strains I, III, IV and V will also split isodulcitol, in which character they differ from strain II, which is completely negative towards this medium. All strains are inactive in Simmons medium, and accordingly show no growth on the Simmons tube. All our strains thus show the same cultural characters with the exception of the lesser disagreement in isodulcitol between strain II and the others.

It will be obvious, that all our strains behave like the common *B. coli* with the exception of their behaviour toward lactose. It is indeed their conduct in this medium which justifies the grouping of these bacteria in a group of microbes separate from ordinary *B. coli*. It will consequently be of considerable interest to study the deficient power of fermentation of lactose in these bacteria. We have namely often observed, that the colonies may show a shade of acid color, when left for some days on a lactose-plate. This made us take up the question, whether the lactose-splitting faculty of these microbes were not latent in the bacteria so that it might be brought about again through successive generations in a lactose medium. Lactose-peptone water was chosen for the experiment and all our strains were carried from one generation to the next in this medium after a growth of twenty-four hours in

each tube of lactose-peptone water (1 per cent lactose in 1 per cent peptone added 0.5 per cent sodium chloride). The result of this experiment was, that two of the strains were able to split the lactose after three days of continual growth in the lactose solution, while two had gained this faculty after nine days of growth and one strain after eighteen days of growth.

It is thus obvious that the absence of the faculty of splitting lactose is really not absolute; this faculty is still present in the

TABLE 3

Agglutination in sera from patients

STRAIN	SERUM I	SERUM II	SERUM III	STRUM IV	SERUM V
I	160	160	640	160	
II		320	640	320	640
III			320	320	
IV				320	
V					640

TABLE 4

Agglutination after absorption in sera from patients

STRAIN	SERUM II AFTER ABSORPTION WITH:		SERUM III AFTER ABSORPTION WITH:			SERUM IV AFTER ABSORPTION WITH:			
	Strain I	Strain II	Strain I	Strain II	Strain III	Strain I	Strain II	Strain III	Strain IV
I	20	20	10	10	10	40	40	40	40
II	20	20	20	20	20	80	80	80	80
III			10	10	10	80	80	80	80
IV						80	80	80	80

bacterium as a latent power, which can easily be trained to its full extent. Whether or not this phenomenon should be called a true dissociation or only an adaptation is of minor importance. The ease, with which it is brought about, seems to indicate, that the eventual "loss" of the power to split lactose cannot have taken place far back in the evolution of these bacteria, but must on the contrary be considered a quite recent happening. We will deal with this phenomenon in the discussion of the species of our strains.

It is already mentioned under the histories of our patients, that the blood tests from the patients agglutinated the homologous strains.   The etiological proof of the relationship between bacteria and disease is thus brought about.   It is, however, of greater interest, that all our strains agglutinated in all the heterological sera, thus indicating the serological agreement of all our strains (table 3).   It will be seen from this table, that all the strains show about the same agglutination in the different sera.   When all strains are not tested in all sera, the reason for this fact is to be

TABLE 5

Agglutination in immune serum from rabbit produced with strain II

STRAIN	BEFORE ABSORPTION	AFTER ABSORPTION WITH:			
		Strain I	Strain II	Strain III	Paracoli
I	5,120	200	200	200	5,120
II	10,240	400	400	400	10,240
III	10,240	400	400	400	10,240
Paracoli	80	0	0	0	10

TABLE 6

Agglutination in immune serum from rabbit produced with strain II

STRAIN	BEFORE ABSORPTION	AFTER ABSORPTION WITH:	
		Strain II	Strain IV
II	6,400	400	400
IV	6,400	400	400
V	6,400		

found in the difference of time of the isolation of the different strains.   All strains were not isolated at the making of each blood test.

The serological agreement between the strains is also seen from table 4, which shows the agglutination test after absorption of the sera with the different strains.   An immune serum produced in a rabbit with intravenous injections of killed emulsions of strain no. II shows still better the complete agreement between all five strains (tables 5 and 6).   The tables are based on investigations

with an interval of time of two months, which explains the different titers in the two examinations.

This serum thus agglutinates all our five strains, and the titer is the same in all strains after absorption. On the other hand, a different non-lactose splitting organism, a "para-colon" bacillus, showed only a very weak agglutination in this serum, and no faculty of absorbing agglutinins. We have consequently to deal with five strains of organisms of the same cultural aspect, isolated from different patients, at different time and in different localities, and all having the same serological characters. It will even be correct to say that all these microbes constitute a serological entity.

None of the five strains showed a serological reaction in sera from parathypohid A or B. *B. Aertrycke* or *B. Gaertner*, i.e., they were negative to all of the Salmonella sera. All serological reactions were the same before and after the change of the strains from non-lactose splitting into lactose fermenting organisms.

DISCUSSION

The five strains of bacteria described agree culturally with the type that Morgan and Ledingham have named type XII. It will here be appropriate to make an examination of the types of bacteria studied by Morgan and Ledingham. One will see from table 1 that these authors include in their scheme microbes that obviously are very slightly related to each other. Thus we find in table 1 "Type Flexner" including microbes that do not produce gas from the carbohydrates, but only acid. This type must be considered as dysentery bacilli and should accordingly not be grouped with gasproducing types. The last type in table 1 is obviously an *Alcaligenes fecalis* and distinctly different from the other types.

When everything is taken into consideration, Morgan and Ledingham have not held the opinion that all the types mentioned by them should necessarily be related to each other. They have only put together the type of bacteria they found during their study of summer diarrhea in children and it would accordingly be incorrect to include all these types under the name of Mor-

gan's bacilli, since several of the microbes in Morgan and Leding-
ham's scheme are already known under other names.   All these
latter types should be excluded from the name of Morgan's bacilli,
and we will have only four types to work with, and all these four
types seem to be fairly closely related, and are not known under
other names.   These types are Morgan and Ledingham's types
I, X, XII, and XIV.   These types might naturally be named
according to their increasing power of fermentation, so that type
I will be the first, type X the second, and so forth as set out in
table 6.   The five strains described in this paper will then be
included in Morgan's type III.

Our strains differ from the Escherichia in that they lack the
faculty of splitting lactose directly on isolation from feces.   On
the other hand they differ from the Salmonella types in their ac-
tivity against sucrose and in their faculty of producing indol and
their negative behavior in the Simmons medium.   But all these
characters that distinguish from the Salmonella types, bring
our strains close up to the Escherichia types.   The sole member
of the Salmonella types that does not grow on the Simmons med-
ium is the paratyphoid A, which, however, is distinct from our
strains in other characters.

As to a generic name for the microbes included in the types
described by Morgan, one will not find any such in any textbook.
One is aware of the fact, that Morgan's type I, by the American
commission of bacteriological generic names is placed with the
Salmonella types and called *Salmonella Morgani*.   Thjøtta (1918,
1920, 1928) has protested against this name and called attention
to the fact that this microbe is not a Salmonella, but bears all the
characters of the Escherichia, despite its poor fermentative facul-
ties.   In this conception he is supported by d'Aunoy (1929).
He accordingly proposes the name of *Escherichia Morgani* for
Morgan's bacillus no. I.

The argument of Thjøtta as to the generic name of Morgan's
type I is still more justified, when we are dealing with the other
types in Morgan's scheme, and especially the types isolated from
our patients.   The only character that excludes these strains
from the typical Escherichia is the lack of the power of lactose

fermentation. When it is now shown that this faculty really is not absolutely lacking, but is latent in the body of the microbes and can be brought about very quickly, it must be obvious that these strains must be types of Escherichia. The new strains, i.e., the lactose splitting strains, derived from the non splitting strains, show no other difference from the mother strains, they agglutinate to the same extent in the immune sera and show the same fermentative characters in all other media. The type III of Morgan is really altered into an Escherichia (into *B. coli-communis*).

The serological agreement between our five strains is of the greatest interest, in that it shows the homogenicity of these five strains from very different sources. The serological examination of our strains also show, that they have nothing whatever in common with the Salmonella types, not agglutinating in any

TABLE 7

	LACTOSE	GLUCOSE	MANNITOL	SUCROSE	DULCITOL
Type I......................	0	A + G	0	0	0
Type II.........	0	A + G	A + G	0	0
Type III......	0	A + G	A + G	A + G	0
Type IV..	0	A + G	A + G	A + G	A + G

serum from these types, while none of the Salmonella types show the slightest agglutination in the immune serum from our strains.

Further, this investigation clearly shows, that Morgan's bacilli will call forth agglutinin production, when they are really infective invaders. This strengthens Thjøtta's opinion as to the strains isolated by him and others from the feces in cases of colitis, where no agglutinin production could be detected, even in the fatal case. In Thjøtta's case of septic infection with the microbe cultivated from the blood, and in Jervell's two cases of urinary infection a rich production of agglutinins were demonstrated. Thus it seems to be a safe conclusion, that a case of any disease, where the Morgan's bacilli possibly may be regarded as the etiologic agents, will show agglutinin production against the isolated strain, if this really is the invader.

CONCLUSION

1. In this paper 5 cases of urinary infections are described, and shown to be caused by the type of bacteria described by Morgan and Ledingham under the type number XII. In the blood of the patients, agglutinins were demonstrated both for the homologous and for the heterologous strains. It is thus proven, that these strains were the etiologic agents in the disease described.

2. The five strains described agree completely with Morgans type XII. They are Gram negative, slightly motile rods, that split mannitol, glucose and sucrose with production of acid and gas, while they do not attack lactose and dulcitol. They all produce indol. All five strains make a serological unity, as they are all agglutinated to the titer limit in an immune serum produced by one of them, and they absorb the same amount of agglutinins from this serum.

3. All five strains possess a latent faculty of fermenting lactose. When grown in a lactose-containing medium they can all be trained to split this sugar so intensely, that they will alter the reaction of the Drigalsky plate and present themselves as acid colonies, while the colonies were all alkaline on isolation and while the stock culture still remains alkaline on streaking on the same medium.

4. In the present paper the author proposes to modify Morgan and Ledingham's scheme and let this only include microbes that are not found under other names. The earlier type I of the authors will remain type I and the other types will be arranged according to their increasing fermentative power. The five strains described in this paper will consequently be gathered in type III.

5. All the different types of the Morgan bacilli are considered atypical types of *B. coli* and should be named *Escherichia Morgani, I, II, III, IV*.

REFERENCES

BAHR AND THOMSEN 1912 Meddelelser fra det kgl. Veterinaer og landbohøi-
    skoles serumlaboratorium, Nr. 19.
D'AUNOY, R. 1929. Amer. Jour. Med. Sci., **178**, 834.

DICK, G. F., DICK, C. H., AND WILLIAMS, J. L. 1928 Amer. Jour. Dis. Child.,
    35, 955.
HAVENS, L. C., AND RIDGWAY, C. 1929. Jour. Prevent. Med., 3, 159.
JERVELL, A. 1928 Norsk. Mag. f. Laegev, p. 1246.
JUNGMANN, P., AND NEISSER, E. 1917. Med. Klinik, Nr. 5.
KINDBORG, E. 1918 Berliner Kl. Woch., Nr. 4.
MACKENZIE, G. M., AND BATT, L. N. 1930 Jour. Immunol., 19, 371.
MORGAN, H. R. 1906 Brit. Med. Jour., 1, 908.
MORGAN, H. R. 1907 Brit. Med. Jour., 2, 16.
MORGAN, H. R., AND LEDINGHAM, J. C. G. 1909 Proceedings of the Royal So-
    ciety of Medicine, Part II, Epidemiological Section.
ØHRUM AND BAHR 1910 Meddelelser fra det kgl. Veterinaer og landbohøiskoles
    serumlaboratorium, Nr. 5.
SELIGMANN, E. 1917 Centralbl. f. Bakt., I Abt., Orig., 79, 71.
THJØTTA, TH. 1918 Med. Rev. of Revs., 24 4.
THJØTTA, TH. 1920 Jour. Bacteriol., 5, 67.
THJØTTA, TH. 1928 Norsk Mag. f. Laegev., p. 583; Jour. Infec. Dis., 43, 349.

STREPTOCOCCI WHICH GROW AT HIGH TEMPERATURES

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INTRODUCTION

Although streptococci have been exhaustively studied, comparatively little work has been done on streptococci which grow at relatively high temperatures. Orla-Jensen (1919) has described *Streptococcus thermophilus* which grows actively at temperatures from 45 to 50°C. and has also called attention to the fact that certain other streptococci grow at 45°C. and above. Aside from the work of Orla-Jensen, very little has been published by other workers dealing with the streptococci which grow at higher temperatures, although a number of investigators have studied certain streptococci which survive pasteurization and other heat treatments of milk and milk products.

The object of the present work was to obtain more definite information concerning streptococci from milk and other sources which are able to develop rapidly at temperatures too high for the growth of pathogenic streptococci, *Streptococcus lactis* and many of the other common streptococci.

In all, 294 cultures of streptococci which grow actively at 45°C. have been studied.

METHODS

The method used in the isolation of cultures was to warm samples of raw milk and flasks of sterile milk to which samples of manure, human feces, ice cream, or cheese had been added, quickly in a waterbath to a temperature of 45°C. and then incubate at this temperature for twenty-four hours. At the end of this period the milk was plated on lactose agar and cultures isolated from the pre-

vailing types of colonies which developed. To ensure pure culture isolations, each colony was observed under the microscope before transferring to litmus milk to see that no other colony might be picked at the same time. The litmus milk was heated to 45°C. before incubation and only those cultures which grew at this temperature were studied further. Gram stains were made on each of the cultures to note chain formation and as a check on the purity of the culture. All of the cultures were isolated in this way with the exception of a majority of the cultures of *Streptococcus fecalis* and *Streptococcus glycerinaceus* which were obtained from one-day-old Swiss cheese.

The hemolysis of blood was determined by plating (not streaking) the cultures on meat-infusion horse-blood agar (pH 7.6) and incubating at 37°C. for forty-eight hours. A study was made of litmus reduction in milk, the most essential point of which is whether the reduction takes place before or after the curdling of the milk. The final pH was determined by the colorimetric method after the cultures had been incubated in 1 per cent glucose broth at 37°C. for fourteen days. Cultures which were grown in 4 per cent peptone solution for forty-eight hours at 37°C. were tested with Nessler's reagent for the production of ammonia. The temperature growth limits were determined in litmus milk. In each instance the culture was cooled or heated, immediately after inoculation, to the temperature at which it was to be incubated.

The ability of the cultures to ferment carbohydrates and related substances was tested with arabinose, glucose, maltose, lactose, sucrose, raffinose, inulin, mannitol, glycerol, and salicin. In order to safeguard against changes of the fermentation test substances in the sterilization process, each substance was sterilized separately by filtration as a 10 per cent solution in distilled water and added to sterile beef extract-peptone broth so as to obtain a 1 per cent solution of the substance to be tested. Controls were run as a check on the fermentations. In each instance the cultures were grown in a 0.1 per cent glucose beef extract-peptone broth for eighteen to twenty-four hours before inoculation into the various substances. Tests for action on starch were made by the use of

starch agar plates.   The cultures were plated with this medium in proper dilution (not streaked) and the plates were then flooded with iodine solution after three days' incubation at 30°C.

The resistance of the cultures to pasteurization was tested by placing 1 cc. amounts of cultures, which had been grown in milk for two days at 37°C., into 10 cc. amounts of sterile milk, previously heated to 62.8°C., and holding the tubes at this temperature for thirty minutes.   Plate counts upon lactose agar were made from the heated samples immediately before and after the pasteurization process in order to obtain an idea concerning the relative rates of destruction of the organisms at this temperature.

EXPERIMENTAL

The groupings which we have made in this study are based on hemolysis of blood; growth at 10°, 45°, and 50°C.; the production of ammonia from peptone; action on litmus milk; and the liquefaction of gelatin; supplemented by the fermentation of carbohydrates and related substances.

The cultures studied are considered under four main groups: (1) *Streptococcus thermophilus*, Orla-Jensen, which appears to represent a homogeneous collection of organisms belonging definitely to one species; (2) the "bovis group," containing the closely related *Streptococcus bovis* and *Streptococcus inulinaccus;* (3) the "fecalis group," in which we have included *Streptococcus fecalis* and *Streptococcus glycerinaceus*, and (4) the "liquefaciens group" containing *Streptococcus liquefaciens* and *Streptococcus zymogenes* because of certain characteristics which they appear to have in common.

All of the cultures isolated have been identified as belonging to previously described species.   We have also been successful in bringing together the valuable works of Orla-Jensen (1919) and of Ayers and his co-workers (1922) (1923) (1924) in the cases in which these investigators apparently dealt with the same groups of organisms.

Streptococcus thermophilus

Seventy-six of the 294 cultures studied belong to the *Streptococcus thermophilus* group.   This name has been applied to these

cultures because our observations conform closely to the descriptions of *Streptococcus thermophilus* as given by Orla-Jensen.

Hucker (1928) has described cultures of *Streptococcus thermophilus* which were isolated from pasteurized milk. His fermentation results correspond to those given in table 1. He classes all streptococci which grow between 38° and 45°C.; ferment sucrose, but not maltose, dextrin nor salicin, and which curdle milk with slight reduction of litmus as *Streptococcus thermophilus*. He did not test for temperature growth limits nor production of ammonia.

TABLE 1

Streptococcus thermophilus

SOURCE OF CULTURES	NUMBER	HEMOLYSIS OF BLOOD	LITMUS REDUCED BEFORE CURDLING	FINAL pH IN GLUCOSE	GROWTH			NH₃ FROM PEPTONE	FERMENTATION OF:										
					10°C	45°C	50°C		Arabinose	Glucose	Maltose	Lactose	Sucrose	Raffinose	Inulin	Starch	Glycerol	Mannitol	Salicin
Raw milk	55	Gamma	−	4 3	−	+	+	−	∓*	+	−	+	+	∓†	−	−	−	−	−
Ice cream	21	Gamma	−	4 2	−	+	+	−	−	+	−	+	+	∓‡	−	−	−	−	−

* Sixty-nine per cent of these cultures changed arabinose from pH 7.0 to 6.6.

† Thirty-four per cent of these cultures fermented raffinose actively while 58 per cent changed the reaction of the medium from pH 7.0 to 6.6, and the other 8 per cent were negative.

‡ Sixty-two per cent of the ice cream cultures fermented raffinose while 38 per cent did not.

Some of his cultures gave a Beta hemolysis and some fermented maltose and grew on plain beef extract-peptone agar.

Robertson (1927) described *Streptococcus thermophilus* but his cultures do not correspond to the cultures which we have reported in table 1. From work which we have done with some of the cultures which Robertson studied, it appears that his cultures belong to the *Streptococcus glycerinaceus* type, since they grow at 10°C.; do not grow at 50°C.; ferment salicin, maltose, glycerol, and mannitol; and produce a larger colony than does *Streptococcus thermophilus*.

The outstanding characteristics of *Streptococcus thermophilus*

as given in table 1 are as follows: Slight reduction of litmus after coagulation of milk; active growth at 50°C.; no growth at 10°C.; only slight growth at room temperature; no fermentation of maltose, inulin, salicin, mannitol, or glycerol; fermentation of glucose, lactose and active fermentation of sucrose; and slight fermentation of raffinose and arabinose.   None of the cultures grew on beef extract-peptone agar without the addition of a fermentable carbohydrate.   The colonies, even when not crowded, were of "pinpoint "type on lactose agar.   Long chains were formed in milk.

All of the cultures in each of the groups reported in this study resisted pasteurization at 62.8°C. for thirty minutes, but cultures belonging to the *Streptococcus thermophilus* group showed a higher percentage survival.

The bovis group

Ayers and Mudge (1923) described two types of *Streptococcus bovis*, varieties "A" and "B" respectively.   Orla-Jensen (1919) suggested that the cultures of *Streptococcus bovis* studied by him might be called types "A" and "X," but he classified the "X" type as *Streptococcus inulinaceus*, based on the fact that the type "X" fermented inulin and raffinose, frequently starch and mannitol, but not arabinose.   His strain "A" differed from Strain "X" in that it fermented arabinose and hydrolyzed starch actively, but usually failed to ferment inulin.

Our results, recorded in table 2, show that *Streptococcus bovis* does not hemolyze blood; that it does not reduce litmus before curdling the milk; and that it does not grow at 10°C. nor at 50°C., but does grow at 45°C.   It ferments arabinose, glucose, maltose, lactose, sucrose, raffinose, starch, and salicin; sometimes mannitol and inulin; but fails to ferment glycerol.   The cultures tested do not produce ammonia from peptone.

These results are the same as those obtained by Ayers and Mudge (1923) in their study of *Streptococcus bovis*, except that they did not test arabinose, maltose, glycerol, and the growth at high temperatures.   They also noted a weak hemolysis of blood which was not detected in our cultures.   Our results also confirm the work reported by Orla-Jensen (1919).   He pointed out that

the typical *Streptococcus bovis* ferments arabinose, has capsules in milk, and hydrolyzes starch, while *Streptococcus inulinaceus* does not ferment arabinose, readily ferments inulin, hydrolyzes starch only weakly, and does not form capsules in milk. With the cultures studied by us, this was found to be true. In other respects the two strains are found to be practically identical, so far as revealed by the tests applied in this study.

TABLE 2

Bovis group

SOURCE OF CULTURES	NUMBER	HEMOLYSIS OF BLOOD	LITMUS REDUCED BEFORE CURDLING	FINAL pH IN GLUCOSE	GROWTH			NH₃ FROM PEPTONE	FERMENTATION OF:										
					10°C	45°C	50°C		Arabinose	Glucose	Maltose	Lactose	Sucrose	Raffinose	Inulin	Starch	Glycerol	Mannitol	Salicin
Streptococcus bovis																			
Milk . .	12	Gamma	−	4 3	−	+	−	−	+	+	+	+	+	+	−	+	−	−	+
Bovine feces .	85	Gamma	−	4.3	−	+	−	−	+	+	+	+	+	+	+	+	−	∓†	+
Streptococcus inulinaceus																			
Milk.	12	Gamma	−	4.3	−	+	−	−	−	+	+	+	+	+	+	−*	−	−	+
Mouth of cow	36	Gamma	−	4.3	−	+	−	−	−	+	+	+	+	+	+	−*	−	∓‡	+
Human feces	15	Gamma	−	4 2	−	+	−	−	−	+	+	+	+	+	+	−*	−	+	+

* Starch was faintly hydrolyzed in some instances.

† Thirty-six per cent of these cultures fermented mannitol while the other 64 per cent were negative or showed a very slight change in reaction.

‡ Eighty-six per cent of those from the mouths of cows fermented mannitol while 14 per cent did not.

While it perhaps would be more logical for the present to designate the arabinose negative, inulin positive type as only a variety of *Streptococcus bovis*, we have followed Orla-Jensen in calling it *Streptococcus inulinaceus*. Of particular interest in this connection is the observation of Ayers and Mudge (1923) that the inulin fermenting type is characteristic of the mouth of cows, while the type isolated from cow feces is characteristically inulin negative. Our results confirm this finding. All of the cultures isolated by us

from the mouths of cows were of the inulinaceus type, while the bovis type was obtained from cow feces.

The fecalis group

As may be seen from table 3, the cultures of this group are divided into two types: (1) *Streptococcus fecalis*, Andrewes and Horder, and (2) *Streptococcus glycerinaceus*, Orla-Jensen. The

TABLE 3

Fecalis group

SOURCE OF CULTURES	NUMBER	HEMOLYSIS OF BLOOD	LITMUS REDUCED BEFORE CURDLING	FINAL pH IN GLUCOSE	GROWTH 10°C	45°C	50°C	NH₃ FROM PEPTONE	FERMENTATION OF: Arabinose	Glucose	Maltose	Lactose	Sucrose	Raffinose	Inulin	Starch	Glycerol	Mannitol	Salicin
Streptococcus fecalis																				
Swiss cheese	10	Gamma	+	4 2	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+
Swiss cheese processed	12	Gamma	+	4 2	+	+	+	+	+	+	+	+	-	-	-	-	$\mp$*	+
Streptococcus glycerinaceus																				
Milk	26	Gamma	+	4 0	+	+	+	+	+	+	+	+	+	$\mp$†	-	-	+	+	+
Swiss cheese	12	Gamma	+	4 2	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
Ice cream	16	Gamma	+	4 1	+	+	+	+	+	+	+	+	+	$\mp$‡	-	-	+	+	+
Bovine feces	38	Gamma	+	4 1	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+

* Seventy-five per cent of the processed cheese cultures actively fermented mannitol.

† Eighty-four per cent of the milk cultures fermented raffinose while 16 per cent were negative.

‡ Thirty-one per cent of the ice cream cultures fermented raffinose while 69 per cent were negative.

cultures of this group were isolated from one-day-old Swiss cheese, processed Swiss cheese, raw market milk which had been held at 45°C. for twenty-four hours, fresh ice cream which had been incubated at 45°C. for twenty-four hours, and bovine feces which were placed in sterile milk and some samples held at 50°C. for twenty-four hours and other samples held at 10°C. for four days.

These two types are similar in that they do not hemolyze blood; reduce litmus before curdling the milk; produce ammonia from peptone; give a final pH in glucose broth of about 4.2; grow at 10°C., 45°C., and 50°C.; ferment arabinose, glucose, maltose, lactose, and salicin; do not ferment inulin nor hydrolyze starch. The members of this group differ in that the fecalis type does not ferment glycerol and usuallý does not ferment sucrose, while the glycerinaceus types ferments both these substances. The glycerinaceus type reduces litmus more rapidly, but does not curdle milk as soon as the fecalis type.

Orla-Jensen (1919) described *Streptococcus faecium* which appears to be similar to *Streptococcus fecalis* as described by Andrewes and Horder (1906), and more fully by Ayers and Johnson (1924). The cultures we have designated as *Streptococcus fecalis* agree with the description as given by Ayers and Johnson for their cultures, and also with Orla-Jensen's description of *Streptococcus faecum*. We are inclined to the belief that these species are the same, and, therefore, that the name *Streptococcus fecalis*, Andrewes and Horder, should be used.

Ayers and Johnson (1924) concluded that *Streptococcus lactis* and *Streptococcus fecalis* are similar if not identical. It is generally conceded that *Streptococcus lactis* does not grow at 45°C., as was shown by Sherman and Albus (1918) and by Orla-Jensen (1919). Since the cultures of *Streptococcus fecalis* studied by Ayers and Johnson were not tested for growth at 45°C., it is felt that it might be possible to use the temperature growth limits as a means of differentiating *Streptococcus lactis* from *Streptococcus fecalis*. Further work on this problem will be reported in another paper, but it may be stated now that other tests have been found which clearly differentiate these two species.

The liquefaciens group

As has been noted by Orla-Jensen, *Streptococcus glycerinaceus* and *Streptococcus liquefaciens* are practically identical in the fermentation of test substances, but the outstanding difference is that *Streptococcus liquefaciens* liquefies gelatin and digests casein while *Streptococcus glycerinaceus* does not. Included in our study

were eight cultures of the liquefaciens type which were isolated from milk.

MacCallum and Hastings (1899) described a new organism from a case of acute endocarditis as *Micrococcus zymogenes*. They stated that the organism had points of resemblance to the pneumococcus and *Streptococcus pyogenes* on the one hand and to the pyogenic staphylococci on the other. While the fermentation tests and blood agar were not in vogue at that time, the description of these workers is excellent in bringing out clearly the cultural and physiological characteristics of the organism. The proteoly-

TABLE 4

Liquefaciens group

SOURCE OF CULTURES	NUMBER	HEMOLYSIS OF BLOOD	LITMUS REDUCED BEFORE CURDLING	FINAL pH IN GLUCOSE	GROWTH			NH₃ FROM PEPTONE	FERMENTATION OF:											LIQUEFACTION OF GELATIN AND CASEIN
					10°C	45°C	50°C		Arabinose	Glucose	Maltose	Lactose	Sucrose	Raffinose	Inulin	Starch	Glycerol	Mannitol	Salicin	
Streptococcus liquefaciens																				
Milk.........	8	Gamma	+	4.2	+	+	∓*	+	−	+	+	+	+	+	−	−	+	+	+	+
Streptococcus zymogenes																				
Human feces.	12	Beta	+	4.3	+	+	−	+	+	+	+	+	+	+	−	−	+	+	+	+

* Seventy-five per cent of these cultures did not grow at 50°C.

sis of gelatin and milk was noted. The strong reducing action of the organism in litmus milk was described, the significant point being noted that the litmus is reduced before acidulation and coagulation of the milk. MacCallum and Hastings also noted that growth was more profuse on glycerol agar than on plain nutrient agar, which indicates that this substance was utilized. In a recent study of this organism Frobisher and Denny (1928) conclude that it should be classified as a streptococcus, perhaps as a variety of *Streptococcus liquefaciens*.

The cultures of *Streptococcus zymogenes* studied by us were all isolated from the feces of one person who was suffering from a temporary but severe attack of diarrhea.

Streptococcus zymogenes (table 4) resembles *Streptococcus lique-faciens* in that both reduce litmus before curdling the milk, liquefy gelatin and casein, have a final pH of about 4.2, grow at 10°C. and 45°C., produce ammonia from peptone, ferment glucose, maltose, lactose, sucrose, raffinose, glycerol, mannitol, and salicin, but do not ferment inulin and starch. These types differ in that zymogenes hemolizes blood and ferments arabinose while the liquefaciens type does not.

SUMMARY

A study was made of 294 cultures of streptococci from milk and other sources which grow actively at 45°C. The most prevalent types were *Streptococcus thermophilus*, *Streptococcus bovis*, *Streptococcus inulinaceus*, *Streptococcus fecalis*, *Streptococcus glycerinaceus*, *Streptococcus liquefaciens*, and *Streptococcus zymogenes*.

Streptococcus thermophilus does not hemolyze blood; reduces litmus milk slightly after coagulation; does not produce ammonia from peptone; grows at 50°C. but not at 10°C.; grows slowly at room temperature; ferments glucose, lactose, and sucrose; does not ferment maltose, inulin, mannitol, glycerol nor salicin; and may or may not ferment raffinose and arabinose. Its resistance to pasteurization is greater than that of the other types studied. When these cultures are grown in milk, long chains are formed.

Streptococcus bovis does not hemolyze blood; does not reduce litmus before coagulation of milk; does not grow at 10°C. nor at 50°C., but does grow at 45°C.; ferments arabinose, glucose, maltose, lactose, sucrose, raffinose, salicin, and hydrolyzes starch; does not ferment glycerol; but may or may not ferment inulin and mannitol.

Streptococcus inulinaceus differs from *Streptococcus bovis* in that it always ferments inulin, but does not ferment arabinose, while starch is hydrolyzed only faintly.

Streptococcus fecalis does not hemolyze blood; reduces litmus before curdling the milk; grows at 10°C., 45°C., and often slowly at 50°C.; produces ammonia from peptone; ferments arabinose, glucose, maltose, lactose, and salicin; does not ferment raffinose, inulin, and glycerol; does not hydrolyze starch; may or may not

ferment sucrose, and mannitol; and does not digest casein nor liquefy gelatin.

Streptococcus glycerinaceus differs from *Streptococcus fecalis* in that it ferments glycerol and always ferments sucrose. Glycerinaceus usually does not curdle milk as rapidly as fecalis.

Streptococcus liquefaciens does not hemolyze blood; reduces litmus before curdling the milk; grows at 10°C., 45°C., and may or may not grow at 50°C.; produces ammonia from peptone; ferments glucose, maltose, lactose, sucrose, raffinose, glycerol, mannitol and salicin; does not ferment arabinose and inulin; does not hydrolyze starch; but liquefies gelatin and digests casein.

Streptococcus zymogenes differs from *Streptococcus liquefaciens* in that it is beta-hemolytic and ferments arabinose.

In view of the present state of our knowledge it might be better to consider *Streptococcus inulinaceus, Streptococcus glycerinaceus* and *Streptococcus zymogenes* as only varieties of *Streptococcus bovis, Streptococcus fecalis* and *Streptococcus liquefaciens* respectively. This, however, is a matter of opinion which should be left to the specialists in taxonomy.

All of the species of streptococci described in this study survive heating for thirty minutes at 62.8°C. in milk.

REFERENCES

ANDREWES, F. W., AND HORDER, T. J. (1906) Lancet, **171**, 708–713; 775–782; 852–855.

AYERS, S. HENRY, AND MUDGE, COURTLAND S. (1922) Jour. Infect. Dis., **31**, 40–50.

AYERS, S. HENRY, AND MUDGE, COURTLAND S. (1923) Jour. Infect. Dis., **33**, 155–160.

AYERS, S. HENRY, AND JOHNSON, W. T., JR. (1924) Jour. Infect. Dis., **34**, 49–53.

FROBISHER, MARTIN, JR., AND DENNY, E. R. (1928) Jour. Bact., **16**, 301–314.

HUCKER, G. J. (1928) New York State Agr. Exp. Sta., Tech. Bul. 134.

MACCALLUM, W. G., AND HASTINGS, T. W. (1899) Jour. Exp. Med., **4**, 521.

ORLA-JENSEN, S. (1919) Mem. Acad. Roy. Sci. et Lett. de Denmark, Copenhagen. Sect. d. Sci., **8**, Ser., **5**, 81–96.

ROBERTSON, A. H. (1927) New York State Agr. Exp. Sta., Tech. Bul. 131.

SHERMAN, J. M., AND ALBUS, W. R. (1918) Jour. Bact., **3**, 153–174.

THE VALUE OF SEROLOGICAL TESTS FOR THE IDEN-
TIFICATION OF PSEUDOMONAS MALVACEARUM

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The chief objects of the present investigation were to make a serological study of various strains of *P. malvacearum* and to determine the specificity of the agglutination test in the identification of this organism, for it was felt that some such test was desirable in view of certain important bacteriological studies in these laboratories concerned with the epidemiology of Black Arm disease under field conditions. To those acquainted with bacteriological investigations of *P. malvacearum*, many difficulties present themselves, more especially with regard to the definite identification of this organism. In the literature, stress has been laid on its cultural characteristics on various media, its optimum temperature, thermal death point, staining reactions etc. but however helpful these may be, it has hitherto been the accepted rule in these laboratories that successful plant inoculation experiments were essential before an organism possessing such characteristics could be positively stated to be *P. malvacearum*. Unfortunately, plant inoculations, to fulfil Koch's postulates, are time consuming, and may give unreliable results for several reasons; chief among these are unfavourable climatic conditions at certain seasons, when low soil temperature and high humidity do not prevail; moreover, the difficulty of obtaining clean seed for raising healthy plants must be borne in mind.

To render this enquiry complete it was deemed advisable to obtain not only strains of *P. malvacearum* from different districts of the Sudan, but also from other countries. Thanks to the assistance of the Curator, National Collection of Type Cultures, Lister Institute, it was possible to obtain strains other than those of Sudan origin.

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The majority of the strains were isolated from different sources in the Sudan and conformed in all morphological and cultural characteristics to the standard description of *Ps. malvacearum*.

ISOLATION AND PROVENANCE

Several strains were supplied by Mr. Massey, Government Botanist; the remainder were isolated from fresh boll lesions.

The strains isolated by, or received from, Mr. Massey are numbered XI to XII, while all strains marked Y were isolated by the writer from green bolls coming from the Gezira (December, 1930):

    (X1) Gubba boll lesion.
    (X2) From Shambat lint twelve months old; pathogenicity proved.
    (X3) From flood water; pathogenicity proved.
    (X4) From suspension of a Shambat lint culture which remained alive in distilled water for a month.
    (X5) Received as a "rough" strain of X2.
    (X6) Received as a "smooth" strain of X2.
    (X7) Gezira flood water; pathogenicity proved.
    (X8) A Shambat strain; pathogenicity proved.
    (X9) Egyptian strain; pathogenicity proved.
    (X10) Kassala strain.
    (X11) An old Shambat strain which was isolated several years ago and has remained constant in cultural characteristics and pathogenicity.

    Y1 to Y9. All these strains were isolated from boll lesions from Gezira Research Farm, Wad Medani.

Method of isolation. After many experiments it was found that the most satisfactory method was as follows: Soak the green boll in absolute alcohol and flame, open under sterile condition, remove small pieces of the infected areas inside with dissecting needles, and place on potato slopes. (The same method is applied to the infected lint.) Growth was usually visible in twenty-four hours, and subcultures on to fresh potato slopes were immediately made. This was found necessary as in many cases a growth of Malvacearum which was visible in twenty-four hours had been completely overgrown in thirty-six to forty-eight hours by other concomitant organisms.

The potato slopes were plated out on agar or McConkey

medium and single colonies picked. It was found extremely difficult in many cases to obtain a pure culture, as Malvacearum often seems to exist in closest association with a Gram-positive bacillus; even, in some cases, what appeared to be single colonies of Malvacearum on subculture showed the other organism.

Other pigmented organisms. It is well known that other yellow pigmented and non-pigmented organisms of the Pseudomonas group are associated with Malvacearum in the plant lesions. Several of these (P1, P2, P3, P4, P5) were isolated with a view to studying the serological association, if any, with Malvacearum.

In addition to the above, the following strains were received from the National Collection of Type Cultures: Campestre (EFS), Campestre (Paine), Malvacearum (EFS), Malvacearum (Trinidad), Hyacinthi, and Pear Blossom.

Antisera were readily prepared by giving rabbits three or four weekly intravenous inoculations of a moderately thick suspension of the organism. Both living, and phenolized emulsions were used for inoculation; there appeared to be no significant difference in the final titer.

Sera were prepared against X3, X5, X11, Y2 (Malvacearum EFS).

Agglutination was carried out by the macroscopic method the tubes being left six hours in a water bath at 52°C. and afterwards on the bench for twenty-four hours. Final readings were made after twenty-four hours, the final titer being taken as the last trace of clumping visible with a hand lens. This was quite easy as the control tubes of the suspension always remained perfectly homogenous.

The results with Malvacearum are given in table 1.

Type of agglutinins. As would be expected from flagellate organisms, both floccular and granular or somatic agglutinins were present in all antisera. The so-called Rough Strain X5 gave a perfect emulsion and like the other strains contained both agglutinins. There was no trace of the "R" agglutinin and the strain is undoubtedly a normal smooth one but with a tendency towards formation of granular looking colonies.

Agglutination emulsions. Twenty-four-hour agar plate cul-

tures emulsified readily in saline.   These were used fresh or, in some cases, made up with 0.5 per cent phenol for floccular agglutination and with alcohol (Bien's method) for granular agglutination.

Agglutinin absorption tests.   Forty-eight-hour agar plate cultures were used, as growth after twenty-four hours is too scanty for the purposes of making strong emulsions.   For the absorption dose at least 15 plates were used for every cubic centimeter of

TABLE 1

SERA	HOMOLOGOUS TITER	ALL STRAINS FROM	
		X1-X11	Y1-Y9
X3	5,000 f., g.*	5,000	5,000
X5	2,500 f., g.	2,500	2,500
X11	2,500 f., g.	2,500	2,500
Y2	2,500 f., g.	2,500	2,500

* f = floccular agglutination.   g = granular or somatic agglutination.

TABLE 2

SERUM	ABSORBED BY	RESIDUAL AGGLUTINATION AGAINST ALL STRAINS
X11	X11	Nil (L50)
X11	X1, X2, X3, X4, Y7, Y8	Nil (L50)
X3	X3	Nil (L50)
X3	X1, X2, X6, X9, X10, Y7, Y8, Y9	Nil (L50)
Y2	Y2	Nil (L50)
Y2	Y9, Y8, Y7, X2, X6, X9, X10	Nil (L50)

serum—doses smaller than this were found insufficient to clear all agglutinins.   The absorbing emulsions were left in contact with the serum for at least six hours, of which one hour was spent at 52°C. and the remainder at room temperature (average 32°C.). As absorptions were in all cases perfectly regular it will be sufficient to give a summary of the results.

It will be seen from table 2 that absorption is completely reciprocal, any one strain removing all agglutinins from a given serum, both for itself and for all other strains.   There would thus

appear to be complete antigenic identity of all the Sudan and Egyptian strains of *P. malvacearum.*

Other strains. P. hyacinthi, Pear Blossom and Campestre (Paine) emulsions were all negative against any Malvacearum serum.

Malvacearum (Trinidad) agglutinated up to full titer of all sera and appeared to be antigenically identical with the Sudanese strains.

TABLE 3

SERUM	EMULSION	TITER
EFS	Y2	125 tr.
EFS	Y9	125 tr.
EFS	X5	125 tr.
EFS	X11	125 tr.
EFS	EFS	5,000
X11	EFS	0
X3	EFS	0
Y2	EFS	0

TABLE 4

SERUM	EMULSIONS			
	P1	P2	P3	P4
X11	0	0	0	0
X3	0	0	0	0
Y2	0	0	0	0

Malvacearum (EFS). This strain gave anomalous results. Cultures on agar did not appear typical of Malvacearum, being white in appearance with an entire absence of stickiness when touched with a loop. The organism grew with great difficulty on potato agar slopes. Like other Malvacearum strains it had no action on carbohydrates. (See table 3.)

It is difficult at present to explain this one-sided agglutination; possibly the American strains differ somewhat from those of the Sudan, or else this strain has altered antigenically with repeated subculture.

Yellow pigmented organisms P1, 2, 3, 4 isolated from green bolls were tested against Malvacearum sera with results as shown in tables 4 and 5.

It is obvious that no kind of antigenic relation exists between these organisms and Malvacearum.

DISCUSSION

There are a few references in the literature to the serological study of members of the Pseudomonas group, in particular the

TABLE 5

SERUM	EMULSIONS		
	X11	X3	Y2
P1	0	0	0
P2	0	0	0
P3	0	0	0

TABLE 6

To exclude the possibility of non-specific (normal) agglutination with Malvacearum

SERUM	EMULSIONS
	Y1 Y2 .... Y9
Normal rabbit (6 specimens) Normal guinea pig (3 specimens) Normal human (6 specimens)	No agglutination with any of the sera (1:25 dilution)

group of yellow pigmented organisms of which Malvacearum is a member.

Brooks *et al.* (1925) showed a remarkable degree of cross relationship between some strains of Campestre, Malvacearum and Phaseoli.

Link and Link (1928) state that the agglutination test can be used to distinguish Malvacearum from other yellow organisms such as Campestre, Phaseoli, Citre, etc., while Malvacearum is more closely related to Sojense than to Campestre. Like Brooks, they place Malvacearum, Campestre, and Phaseoli in one serological group.

The group relationship of Malvacearum scarcely falls within the scope of the present paper but a few agglutinations carried out between Malvacearum and Campestre (Strain EFS) showed some degree of cross agglutination. With Campestre (Paine), Hyacinthi, Pruni, the results were completely negative. Strains of Phaseoli were unfortunately not available.

SUMMARY AND CONCLUSIONS

1. Plant inoculation experiments for the identification of *P. malvacearum* may give misleading results.

2. The agglutination test is a simple and highly specific method for the identification of this organism.

3. All African strains examined fall into one serologically homogenous group.

4. There is no serological relationship between *P. malvacearum* and the yellow pigmented organism commonly found in association with it in the lesions of Black Arm disease.

REFERENCES

BROOKS, NAIN AND RHODES. 1925. Jour. Path. and Bact., **28**, 203.
LINK AND LINK. 1928. Bot. Gazette, **85** (2), 178.

THE RESISTANCE OF DEHYDRATED PNEUMOCOCCI TO CHEMICALS AND HEAT

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It is well recognized that desiccation not only prevents bacterial growth, but under certain conditions leads to the death of many microörganisms. Leaving aside the extreme resistance of spore-bearing bacteria, it is known, however, that even relatively delicate bacteria such as the typhoid bacillus and the pneumococcus, although they may be destroyed in a thin layer from aqueous suspension when slowly desiccated, remain alive after drying to a constant weight when assembled in large masses (Reichel). The survival of the pneumococcus in the dried spleen of mice dead from infection of a virulent culture is made use of as current procedure in conserving both cultures and virulence.

The condition of "suspended animation" of dehydrated bacteria is in itself a matter for interesting speculation. Of more tangible import is the fact that the presence of water is necessary to stain bacteria (Churchman (1928)) and that dehydrated bacteria are relatively unaffected by many active disinfectants in anhydrous alcoholic solution (Ford (1927)). It seems less well known that desiccated bacteria are also relatively very resistant to heat.

Our interest in submitting desiccated pneumococci to a new and more complete analysis as regards resistance to chemicals and heat, lay first in affording another basis of comparison between bacteria and certain filterable viruses, since the latter are also known to withstand desiccation. And, again, it was thought to be interesting to test the relative resistance of colonial dissociants ("Rough" and "Smooth" forms) of a given pneumococcus to heat

and chemicals. Such dissociants are of particular interest since they are correlated with differences in virulence.

Organism

The organism used in this work is a Type I pneumococcus. It is a highly virulent strain which has been kept in the laboratory for some time and known as No. 94. One culture from this strain was carried in broth for four to six months and became avirulent. A second culture from this same strain was kept virulent by mouse passage. Mice were regularly killed by 1/100,000 of a cubic centimeter in twenty-four hours. The virulent and the non-virulent cultures used in these experiments came, then, from one original strain. Single cell isolation was not resorted to, as the strain had been plated and purified many times. The virulent strain was repeatedly observed on plating as "Smooth" in colonial form, whereas the avirulent strain was "Rough."

Preparation of dried material

An actively growing broth culture was used to inoculate flasks of infusion broth, at pH 7.8, and these flasks were incubated for from eighteen to twenty-four hours. The broth was removed to large centrifuge tubes and centrifugalized and the sediment collected and placed in a vacuum desiccator at room temperature over calcium chloride until dry. When dry, usually in about two days, the sediment was ground to a fine powder in an agate mortar until there were no particles large enough to be visible to the eye. This dried ground powder reached constant weight after about twenty-four hours in the desiccator.

Amount of dried material used

A small standardized curette was found to contain, when level-full, approximately 4 mgm. of the dried material. One curette load was used for each determination.

Many lots of the dried material were prepared in separate small test tubes which were kept in the desiccator so that the material could be used in the experiments within a week of the time of

preparation. Other tubes were sealed and kept in the dark at room temperature for later tests. The number of viable organisms in the dried material was found by plating to vary within fairly narrow limits during the usual period of ten days to two weeks after drying. In the four milligrams used, there were roughly from 200 to 400 million living organisms.

Preparation of alcoholic solutions of bactericidal chemicals

So-called "absolute" alcohol (99.7 per cent) was added to a large bottle half full of quick lime and left in contact for at least a week. Precautions were taken to protect this alcohol from exposure to air so that it would remain water free. On testing with anhydrous copper sulphate the alcohol appeared to be water free. One gram of the disinfectant to be tested was dissolved in 100 grams of this anhydrous alcohol, (i.e., 125 cc.) so as to obtain a one per cent solution by weight.

Technique of tests

To a plugged and sterilized test tube, 8 by 100 mm., previously drawn out in a flame so as to make a constriction near the middle, a curette load of the dried material was added and, on tapping, this material fell to the bottom of the tube. Approximately 0.75 cc. of the alcoholic solution of the disinfectant was added by a capillary pipette and the tube sealed in a flame at the constriction. The sealed bulb thus formed was incubated for twelve hours at 37°C. Following the treatment period of twelve hours, the bulb tip was flamed and broken open. The contents were removed by a sterile capillary pipette to a sterile, rubber-stoppered centrifuge tube containing at least 5 cc. absolute alcohol. This tube was centrifugalized until it was clear and the sediment was packed in the bottom: the supernatant wash alcohol was decanted and 10 cc. of fresh alcohol added. The sediment was again suspended in the alcohol by shaking and the tube again centrifugalized. This operation was repeated with a second washing with 10 cc. of alcohol. Thus the sediment was washed in three lots of alcohol and the disinfectant thereby almost entirely removed. Following the decanting of the last wash alcohol

the centrifuge tube with sediment was placed in the vacuum desiccator until the alcohol had evaporated, which usually took about an hour.   About 7 cc. of broth was now added to the centrifuge tube and a sterile cotton stopper substituted for the rubber stopper.   The tube was incubated for four days with daily observations.   A tube showing growth was examined microscopically so as to rule out possible contamination.   At the end of the four·day period all tubes not showing growth were reinoculated with a loop of a broth culture of pneumococci and again incubated.   This step acted as a check to show that the broth did not contain enough disinfectant to have a bacteriostatic effect.

Control tests of the destructive effect of chemicals employed on "moist" pneumococci

The chemicals: alcohols, salts of heavy metals, dye stuffs and others, that we have tested on dried pneumococci were all known or suspected to be destructive for most bacteria under their normal conditions of growth.   A control check of the bactericidal effect of most of these materials or cultures in aqueous suspension seemed desirable.   For this purpose the original cultures used to prepare the dehydrated preparations were employed as follows: eighteen to twenty-four-hour cultures in the standardized broth were found on plating to contain 200 to 600 million organisms per cubic centimeter.   Two cubic centimeter amounts of this culture were replaced in separate small tubes and centrifugalized until clear.   The sediments after removal of the supernatant fluid contained, then, something less than 400 to 1000 million as compared with 200 to 400 million employed in dried culture experiments.   In other words there were more moist bacteria used in these tests than dried bacteria.

To each sediment the chemical, when soluble in broth or Ringer's solution, was added in solution in this nutritive fluid in 1 cc. amounts.   Chemicals like alcohol, chloroform, xylol and the essential oils were added to the moist sedimented culture intact. These mixtures were allowed to stand at room temperature for eighteen to twenty-four hours in well-stoppered tubes.   The

tubes were centrifugalized, the supernatants decanted, and 5 cc. of broth were added to the entire sediment. To avoid continued action of the disinfectant the tubes were shaken and 1 cc. of the suspended organisms was added to a fresh 5 cc. tube of broth. Growth was allowed for four days at incubator temperature and, when positive, was checked morphologically.

SURVIVAL OF DESICCATED PNEUMOCOCCI

It is known that the completely desiccated blood or organs of mice dead of pneumococcus infection not only contain living pneumococci for many months, but that these organisms retain their virulence unaltered, (Neufeld and Schnitzer (1928)). Griffith is stated by these authors to have obtained positive cultures by these methods for as long as three years. Our dried preparations, not of infected organs, but of bacteria, have been found to contain living organisms for eighteen months.

There is a marked decrease of living bacteria in the process of desiccation itself however rapidly it be performed. By plating out the original broth culture, and deducting the number of organisms found, also by plating, in the supernatant fluid after centrifugalization, the number of living bacteria present in the sediment may be determined. After complete desiccation the total weight of dried bacteria per cubic centimeter of original culture may be calculated. From this, by plating, the number of living dried bacteria and their percentile relationship to those present in an aliquot part of original culture was determined.

Two estimates of this sort indicated:

1. In a rapid desiccation, two days from original culture to apparent dryness: Constant weight fourth day; 8.62 per cent of Rough pneumococci survived, and 7.28 per cent of Smooth pneumococci survived.

2. In less rapid desiccation, four days from original culture to apparent dryness and constant weight: 0.546 per cent of Rough pneumococci survived; 0.042 per cent of Smooth pneumococci survived.

It is evident that slight variations in technic suffice to affect the resistance of pneumococci to desiccation. A single attempt to

obtain dried organisms in larger numbers by growth in 1 per cent glucose broth, in which it is known that more acid is produced, resulted in a sterile desiccate.

The greater resistance of the "Rough" organisms to desiccation should be recalled in consideration with results presently to be given.

TABLE 1

The destructive effect of various anhydrous solvents on moist and on dried smooth pneumococci*

CHEMICAL EMPLOYED	MOIST CULTURE	DRIED CULTURE
Ethyl alcohol	0*	+*
Amyl alcohol............................	—	+
Butyl alcohol	—	+
Caprylic alcohol.................	—	+
Acetone	0	+
Aniline..........................	—	+
Amyl acetate..........	—	+
Benzene	0	+
Benzaldehyde......	0	+
Carbon tetrachloride	0	+
Chloroform..	0	+
Ether	0	+
Ethyl acetate	0	+
Ethylene chloride.	0	+
Petroleum ether	0	+
Toluene	0	+
Xylene	0	+

* The plus sign (+) indicates that the pneumococci survived the exposure, as shown by growth; the "0" sign indicates complete destruction with negative growth; the dash (—) indicates "not tested."

THE EFFECT OF VARIOUS ANHYDROUS SOLVENTS ON MOIST AND DEHYDRATED CULTURES OF PNEUMOCOCCI

In table 1 the effect of various anhydrous solvents on moist and on dehydrated preparations of "Smooth" pneumococci is shown.

It is evident that none of the solvents tried destroyed dehydrated pneumococci whereas all were completely destructive for moist cultures of pneumococci. The series of solvents were also tested after mixture with equal parts of anhydrous ethyl alcohol and were likewise without effect on the dried bacteria.

It is evident that only such other bactericidal chemicals could be tried on dried bacteria as are soluble in one of the anhydrous solvents. As a matter of fact we have tested only certain ones that are soluble in anhydrous ethyl alcohol.

TABLE 2

The destructive effect of various compounds of mercury in solution of ethyl alcohol on moist and dried pneumococci in both "S" (virulent) and "R" (avirulent) forms

COMPOUND AND DILUTION	MOIST		DRIED	
	"S"	"R"	"S"	"R"
Mercuric chloride:				
1 per cent	0	0	+	0
0.1 per cent	0	0	+	0
0.01 per cent	0	0	+	+
0.001 per cent	0	+	+	+
Mercuric cyanide:				
1 per cent	—	—	0	0
0.1 per cent	—	—	0	0
0 01 per cent	—	—	+	0
0 001 per cent	—	—	+	±*
Mercuric iodide:				
1 per cent.	—	—	0	0
0 1 per cent	—	—	0	0
0.01 per cent	—	—	+	+
0.001 per cent . .	—	—	+	+
Mercurochrome:				
Saturated†	—	—	0	0
10 per cent	—	—	+	0
1 per cent.. . .	—	—	+	+
0 1 per cent	—	—	+	+

* These dilutions were always run in duplicate; ± indicates that one tube was sterile and the other showed growth.)

† Mercurochrome is only slightly soluble in alcohol. In this series we have employed a saturated solution and percentages of it.

THE DESTRUCTIVE ACTION OF CERTAIN HEAVY METALS ON DRIED PNEUMOCOCCI

Bichloride of mercury is known to be highly destructive for all bacteria in dilutions of from 1:10,000 to 1:100,000 (Zinsser) (table 2).

It is evident, then, that these compounds of mercury are destructive of "R" dried pneumococci, although less so than for the organisms in watery suspension. The virulent strain of our pneumococcus ("Smooth") was more susceptible when moist than the "rough" but much more resistant than the rough when dried.

Silver nitrate is another well known chemical disinfectant which kills cocci under normal conditions in dilutions of 1:1,000 (Zinsser in table adapted from Fleugge, p. 84). It was likewise destructive of our dried pneumococci for the virulent strain in alcoholic solution in similar concentrations (1:1,000). It killed the dried avirulent strain at times, but not regularly, in a dilution of 1:10,000.

The effect of specific pneumococcidal chemicals on moist and dried pneumococci

Ethyl-hydrocuprein (Optochin)[1] has long been known to be actively, and more or less specifically, destructive for the pneumococcus (Morgenroth and Levy (1912). It has been found to sterilize cultures of this microörganism within twenty-four hours in an aqueous dilution in proportions of from 1:400,000 to less than 1:1,000,000 (Morgenroth (1914). We have found our preparation active in aqueous solution on moist cultures diluted 1:100,000. With an old and less active preparation of optochin, the moist "S" strain was killed in higher dilution than the moist "R" preparation.

Sodium desoxycholate[2] is the most active component responsible for the specific lytic effect of bile on the pneumococcus. Kozlonski (1925) found this salt active in broth cultures in a dilution 1:1,000, whereas bile was effective only in a dilution of 1:10. In our control experiments on moist cultures, a 0.02 per cent solution of sodium desoxycholate in broth completely destroyed the pneumococci.

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[1] (Optochin hydrochloride (Zimmer and C. Vereinigte Chinin Fbrik. Frankfort, Germany as employed by Morgenroth) kindly furnished us by Dr. Oswald T. Avery of the Hospital of The Rockefeller Institute was used in our experiments.)

[2] (Desoxycholic acid was also furnished us by Dr. Avery. A sodium salt was obtained by dissolving in NaOH.)

Both optochin and desoxycholic acid are readily soluble in alcohol. As shown in table 3 neither substance dissolved in alcohol has the slightest effect in 1 per cent solution or less on dehydrated pneumococci whether "R" or "S."

TABLE 3

Effect of optochin and desoxycholic acid in alcoholic solution on dehydrated pneumococci

CHEMICAL AND DILUTION	DRIED "S"	DRIED "R"	MOIST "S"
Optochin:			
1 per cent......	+	—	—
0.1 per cent......	+	+	0
0.01 per cent..........................	+	+	—
0 001 per cent.........	+	+	0
Desoxycholic acid:			
1 per cent......	+	+	—
0.1 per cent	+	+	0
0.01 per cent	+	+	—
0.001 per cent	+	+	—

A few controls indicate the destruction produced by these substances in broth on moist cultures.

TABLE 4

Effect of phenol and of iodine in alcoholic solutions on dehydrated pneumococci

CHEMICAL AND DILUTION	DRIED "S"	DRIED "R"
Phenol:		
5 per cent...	+	+
1 per cent......	+	+
Iodine:		
1 per cent........................	+	+
0.1 per cent............................	+	+
0.01 per cent...............	—	+

THE ACTION OF IODINE AND PHENOL IN ALCOHOLIC SOLUTION ON DRIED PNEUMOCOCCI

Both iodine and phenol are recognized disinfectants. Carbolic acid is said to destroy pneumococci in dilution of 1:60. It was found to destroy our moist pneumococci when dissolved in broth or Ringer's solution in a dilution of 1:200. Neither sub-

stance was active in the concentrations tested in alcoholic solution on dried preparations as shown in table 4.

THE ACTION OF DYE STUFFS ON DRIED PNEUMOCOCCI

Many dye stuffs, particularly gentian violet, are known to be very destructive for bacteria and particularly for those that retain the Gram stain. Pneumococci "R" and "S" were destroyed when moist in a dilution of gentian violet of 0.1 per cent; they are then apparently more resistant to this dye than streptococci (Gay and Morrison) although no direct comparison has been made. When tested on dehydrated pneumococci, whether virulent or avirulent, alcoholic solutions of gentian violet and of basic fuchsin had not the least destructive action in 1 per cent solution.

THE ACTION OF ESSENTIAL OILS ON DRIED PNEUMOCOCCI

Omeltschenko (1891) tested the germicidal properties of a considerable number of essential oils on the typhoid bacillus, the tubercle bacillus and anthrax spores. He found their effect due not only to a direct action, but particularly to their vapors and respective vapor tension. The oils of cinnamon, fennel and lavender were most effective. The action of the vapors was much less effective on dried bacteria. Destruction of the bacteria tested was accompanied by the appearance of granules and their failure to stain. Our preliminary tests on the direct destructive effect of arachis (peanut), and of cinnamon oil indicated that their addition to moist cultures was definitely germicidal under the conditions that have been specified. We then tested a number of these essential oils, both alone and admixed with equal parts of alcohol on the dehydrated bacteria.

The following oils were tested:

Arachis	Origanum both Crete and pure
Cedar	Sandalwood
Cinnamon	Tansy
Citronella	Thyme
Clove	Wintergreen

All these oils were inactive on dried pneumococci except clove, tansy and, in one test, cinnamon oil. It may be that this excep-

tional action is due to admixture of water with the oil, which possibility is rendered more likely in view of the fact that a mixture of equal parts of these particular oils with water-free alcohol was, in no case, active on the dried preparations.

EFFECT OF HEAT ON MOIST AND DRIED PNEUMOCOCCI BOTH "R" AND "S" VARIETIES

It is generally accepted that the thermal death point for pneumococcus as given by Sternberg is 52°C. for ten minutes. We

TABLE 5

Thermal death points of "moist" "R" and "S" cultures of pneumococcus, Type I

C.	15 MINUTES		30 MINUTES		1 HOUR	
	"R"	"S"	"R"	"S"	"R"	"S"
52°	+	+	+	+	+	0
56°	+	0	+	0	0	0
60°	0	0	0	0	0	0

TABLE 6

Thermal death points of desiccated "R" and "S" cultures of pneumococcus, Type I

C.	15 MINUTES		30 MINUTES		1 HOUR	
	"R"	"S"	"R"	"S"	"R"	"S"
65°	+	+	+	+	+	+
100°	+	+	+	+	+	+
110°	—	—	+	+	—	—
115°	—	—	+	+	—	—
120°	—	—	0	0	—	—
125°	—	—	0	0	—	—
150°	—	—	0	0	—	—

find no reference to the relative resistance of "R" and "S" strains of this or indeed of other organisms nor is there reference to the effect of heat on desiccated bacterial cultures.

In tables 5 and 6 are given our determinations of thermal death points which have been consistently repeated.

Technic. "Moist" bacteria were heated in the form of 1 cc. of an eighteen to twenty-four-hour culture in sealed tubes, at the temperatures and for the periods indicated.

Dry bacteria were heated in 6 mgm. amounts in sealed dry tubes immersed in water (65° to 100°) or in a dry sterilizer (110° to 150°) at the temperatures and for the periods indicated.

CONCLUSIONS

Pneumococcus Type I in the form of both virulent and avirulent ("Smooth" and "Rough") dissociants is susceptible when grown in broth to the usual disinfectants heavy metals, dye stuffs, anhydrous solvents, phenol and iodine; and to certain more or less specific substances such as optochin and bile salts. In two instances it could be shown (mercuric chloride, optochin) that the "Smooth" organism was more readily killed than the "Rough" form.

When the microörganisms are collected by centrifugalization and rapidly dried to constant weight over $CaCl_2$ a large proportion of the cells are killed, the surviving percentage depending on the technique employed. The surviving pneumococci may continue to decrease in number but some, at all events, survive for as yet undetermined periods—for eighteen months at least.

Desiccated but living pneumococci of both forms "R" and "S" are not killed in the absence of water by alcoholic solutions of the substances described except in the case of the heavy metals (mercury salts, silver nitrate). Dried "S" pneumococci, contrary to the findings in moist cultures, are more resistant to mercuric chloride than the "R" forms.

The thermal death point of moist "R" pneumococci (56°) is distinctly higher than that of moist "S" pneumococci. When the two dissociants are dried they are both resist heating to 115° for 30 minutes but are killed by exposure to temperatures of 120° and above.

REFERENCES

CHURCHMAN, J. W. 1928 Staining Reactions of Bacteria. The Newer Knowledge of Bacteriology and Immunology. University of Chicago Press, Jordan and Falk, p. 19.

FORD, W. W. 1927 Text Book of Bacteriology. Philadelphia, W. B. Saunders Co.

GAY, F. P., AND MORRISON, L. F. 1921 Experimental streptococcus empyema. II. Attempts at dye therapy. Jour. Infect. Dis., 28, 1–17.

KOZLONSKI, A. 1925 Comparative studies of the action on the pneumococcus of bile acids and unsaturated fatty acids found in bile in the form of soaps. Jour. Exper. Med., **42**, 453.

MORGENROTH, J. 1914 Die Chemotherapie der Pneumokokkeninfektion. Berlin, Klin. Wochnscht., **47** and **48**, 1.

MORGENROTH, J., AND LEVY, R. 1912 Berlin, Klin. Wochns., **14**, 663.

NEUFELD, F., AND SCHNITZER, R. 1928 Pneumokokken. Kolle, Kraus, und Uhlenhuth, Handbuch der Pathogenen Mikroorganismen, Fischer, Jena, **4.**

OMELTSCHENKO, TH. 1891 Ueber die Wirkung der Daempe Aetherischer Oele auf die Abdominal-typhus, Tuberkelund Milzbrandbacillen. Centralbl. für Bakt., **9**, 813.

REICHEL, H. 1928 Desinfektions und Sterilisations Lehre. Handbuch d. path. Mikroorganismens, Kolle, Kraus, und Uhlenhuth, Fischer, Jena, **3**, 855.

FILTER-PASSING ANAEROBIC BACTERIA OF THE UPPER RESPIRATORY TRACT IN HEALTH AND DURING ACUTE RESPIRATORY DISEASE

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The observations here reported form part of a study of the nasopharyngeal flora of persons in isolated communities during health and when they were suffering with colds. The results of studies of aerobic flora have been published (Burky and Smillie (1929); Milam and Smillie (1931)). Field studies have been made in southern Alabama, in Labrador, and in St. John, one of the Virgin Islands in the West Indies. In the course of this field work cultures were made of the filter-passing anaerobic organisms of the nasopharynx of normal persons and of those with colds. This report is a summary of the results of this work.

Olitsky and Gates (1920) first isolated a minute microörganism from the nasopharyngeal washings of early uncomplicated cases of influenza. This organism, which they named *B. pneumosintes*, is a cocco-bacillus, non-motile, Gram-negative, very small, and entirely anaerobic. It was first recovered in Smith-Noguchi medium; later, it was grown on blood-agar plates. Other workers subsequently reported the isolation of *B. pneumosintes*; Loewe and Zeman (1921) obtained strains from 3 cases of influenza; Gordon (1922) reported its presence in fourteen of 20 cases of influenza; Detwiler and Hodge (1924) in three of 6 cases, and Lister (1922) in five out of 15 cases of the same disease. Branham and Hall (1921), McIntosh (1922), and Traut and Herrold (1930) reported negative results from influenza studies.

Later, Olitsky and Gates (1922) and Olitsky and McCartney

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(1923) reported the isolation of other filter-passing, anaerobic, Gram-negative organisms from the nasopharyngeal secretions of various persons.   These strains resembled *B. pneumosintes* but could be differentiated from it.   They were found both in normal individuals and in persons with colds.

Olitsky divided these organisms into three main groups and two subgroups.   Each group is distinct from the others, and none of them is pathogenic for rabbits.   These filter-passing strains have been isolated and described by Mills, Shibley, and Dochez (1928), Noble and Brainard (1928), and Levinthal (1928), and have been recovered by them from individuals in health and during colds.   In so far as possible, we have followed Olitsky and Gates' classification and technique in our field work.

MEDIA

Throughout this study, hormone broth made according to the method of Fisk and Burky (1925) was used.   For a solid medium from 1.5 to 2 per cent agar was added.   In Labrador it was necessary to substitute the entire muscular structure of sheep for the beef heart used in the original formula.   This medium, when used later, parallel with beef-heart medium, was found to be entirely satisfactory.   In the West Indies, because of the absence of ice and the distance from the source of supply, the beef heart when received was always well autolyzed and in many cases putrid.   This produced the best medium used throughout the study, both for this group of organisms and for the ordinary aerobes.

TECHNIQUE

Nasopharyngeal washings.   It was necessary to modify the original methods somewhat in order to meet the exigencies of field conditions.   Our method was as follows:

By means of a 20 cc. syringe tipped with a rubber urethral catheter the nasopharynx is washed with hormone broth.   The catheter tip fits fairly well into one nostril and the broth is forced into the nasopharynx, the patient having been previously instructed to allow the fluid to run out of the mouth into a sterile pus basin   Following the passage of the

fluid, the patient is instructed to cough and spit out all the loose material in the mouth and nasopharynx. The fluid thus collected is transferred to a sterile glass-stoppered bottle containing glass beads and kept at room temperature until filtration is begun. The bottle is then shaken, and the contents transferred to a Berkefeld filter (V) and filtered at a negative pressure of at least 50 cm. of mercury. The filters are discarded after five filtrations.

The filtrate was planted in two media:

a. One-per-cent glucose hormone agar, to which rabbit blood is added (5 per cent). To 12 cc. of the media at 42°C., 3 cc. of the filtrate is added and a pour plate made.

b. Freshly boiled and cooled hormone broth (10 cc.) is inoculated with 3 cc. of the filtrate. Five per cent rabbit blood is added and the tube sealed with vaseline. The plates are incubated at 37°C., in a

TABLE 1

Comparison of growth of anaerobic filter-passing organisms in various types of media

	POSITIVE GROWTH	NEGATIVE GROWTH	CONTAM-INATED	NOT DONE
Blood-agar surface plate................	10	8	2	
Blood-agar pour plate..................	13	7	0	
Hormone blood broth...................	9	6	0	5
Smith-Noguchi medium......	5	7	4	4

Brown anaerobic jar for at least five days; the broth tubes are incubated at the same temperature for one to two weeks and then subcultured on blood-agar plates and incubated in anaerobic jars.

Control aerobic cultures were made in the manner described above and were run in parallel series.

Attempts were made to inoculate blood-agar plates on the surface as recommended by Olitsky, but this procedure is not feasible under field conditions. Smith-Noguchi media could not be used because of the impossibility of obtaining suitable ingredients. The pour plates offer a number of advantages: (1) a larger inoculum may be used, (2) chance contaminations are promptly isolated, (3) separate colonies are obtained on first inoculation, and they are both deep and on the surface, (4) deep colonies show

distinctive types of colony formation and sometimes show hemolysis, which is not seen in surface colonies.

The technique which we used in the field for the cultivation of these organisms was employed with a series of filtrates. Parallel series of cultures were made of the same filtrates, using the methods recommended by Olitsky and Gates.

The results of these parallel series of cultures are given in table 1. The results obtained with the pour-plate method combined with the blood-hormone broth were very satisfactory (see table 1) and this technique has been followed in all our field work.

ALABAMA STUDIES

Our first field studies were conducted in Andalusia, Alabama. Most of the nasopharyngeal washings were collected from a group of school children in an isolated community, at 20 miles' distance, and brought to the laboratory; others were obtained from various sources. Material was secured from normal persons, individuals with colds, and from cases of clinical influenza.

Delay in inoculation of the nasal washings. Under field conditions, the materials to be cultivated must often be transported for considerable distances, which results in a delay in inoculation of the media with the nasal washing. In many instances three hours elapsed before filtering was begun. In our initial work in Alabama the cultures were uniformly negative. In order to determine if these organisms were actually absent from the nasopharynx of people in isolated communities or whether our technique was at fault, the following experiment was performed:

Four young men of the isolated area, from whom cultures had shown negative results, were given a nasopharyngeal washing in the usual way at their homes. This material was transported 20 miles over very bad roads and after three hours had elapsed the filtrate was finally inoculated into media and placed in the incubator. On the following day the same four persons were brought to the field laboratory, and nasopharyngeal washings were made. The washings were allowed to stand for three hours at room temperature before they were filtered. The filtrate was inoculated into media and placed in the incubator. All of the first set of cultures were negative; three of the four cultures of the second set were positive.

These results indicate that the time interval between nasal washing and inoculation of the filtrate is not so important a factor in destruction of the organisms as the factor of unavoidable shaking which accompanied the long transportation. Later, in the studies made in the West Indies, nasal washings were allowed to stand for as long a period as six hours, and were then filtered and cultured. The results were excellent.

Results of the Alabama studies. Nasopharyngeal washings were obtained from twenty individuals; five from normal persons, seven from persons with common colds, and eight from persons with influenza. In the influenza group, one washing was taken during the first day of the disease, one during the second day, five during the third day, and one during the sixth day. Anaerobic filter-passing strains were recovered from 65 per cent of the individuals examined (see table 2). *Bacterium pneumosintes,* the diagnosis of which was confirmed by agglutination, was recovered from one person with a cold. Organisms suggestive of *B. pneumosintes* were recovered from two of the five normals, from three of the seven persons with colds, and from two of the eight persons with influenza. Lack of sufficient growth prevented confirmation by agglutination.

During the course of this study there developed in River Falls, a nearby village, a well-defined epidemic of clinical influenza, which attacked the majority of the small population. Washings were taken from four well-defined cases early in the disease. Pneumosintes-like organisms were recovered in two instances but agglutination tests were not possible, owing to the lack of sufficient growth.

In seven instances, we obtained an organism which resembled *B. pneumosintes* in morphology and colony formation. These strains also resembled group II of Olitsky's classification, and we were not able to place them definitely in one category. It will be noted that several nasal washings contained two or three different types of organisms.

No growth was obtained from one of the five normal persons, from one of the seven persons with colds, and from five of the eight persons with influenza.

TABLE 2

Summary of findings in persons examined for filter-passing anaerobes in the nasopharynx—Alabama

AGE	CLINICAL CONDITION	BACTERIOLOGICAL FINDINGS (OLITSKY'S CLASSIFICATION)	REMARKS
35	Normal	*B. pneumosintes* or II, I	
19	Normal	*B. pneumosintes* or II, I, IIIa	
25	Normal	I, III	
25	Normal	Negative	
25	Normal	Ia	
37	Caught cold night before after chilling motor trip	*B. pneumosintes*, III	Agglutination positive with pneumosintes serum
21	Cold, of 2 weeks' duration, symptoms negligible at time	*B. pneumosintes* or II	
13	Cold for indefinite period	*B. pneumosintes* or II, I, III	
12	Cold for indefinite period	Negative	
13	Cold for indefinite period	I, II	
12	Cold for indefinite period	*B. pneumosintes* or II, III	
25	Cold; influenza?	I, Ia, III, IIIa	
19	Influenza? 3rd day of disease	Negative	These three cultures were gathered at a distance of 10 miles. Anaerobic procedures were complete within normal time limits
40	Influenza? 3rd day of disease	Negative	
15	Influenza? 6th day of disease	Negative	
25	Influenza; 3rd day of disease. Temperature; leukopenia	*B. pneumosintes* or II, I, III	These four cultures were gathered in River Falls, a distance of 5 miles. Anaerobic procedures were complete in normal time limits
21	Influenza; 3rd day of disease. Temperature; leukopenia	Negative	
19	Influenza; 3rd day of disease. Temperature; leukopenia	Negative	
28	Influenza; 1st day of disease. Temperature; leukopenia	*B. pneumosintes* or I1	
25	Influenza; 2nd day of disease. Temperature: leukopenia	II, IIIa	

The types of organisms recovered are similar to those found elsewhere by previous investigators (see table 2).

LABRADOR STUDIES

In the Labrador studies our results were disappointing. The same cultural methods were followed which had been used in Alabama. Cultures of nasopharyngeal washings from twenty-one normal individuals and 17 cases of sporadic colds were negative. We believe that these negative results were not due to the absence of filter-passing anaerobic organisms in the nasopharynx of the native, half-breed, and Indian population, but to some fault in our technique.

We had made two minor modifications of our Alabama technique in carrying out the Labrador studies. In Alabama, beef hearts had been used in making the media; in Labrador, we used the entire muscular structure of a sheep. In Alabama, the negative pressure required for filtration of the washings through the Berkefeld filter had been secured by the ordinary Chapman water pump; in Labrador, it was necessary to substitute for the Chapman pump some other means of obtaining negative pressure. A hand pump, because of its size and portability, was selected. This pump drew the material through the Berkefeld filter easily, and seemed satisfactory in every way.

In order to determine whether or not our failure to cultivate filter-passing anaerobes in Labrador was due to faulty technique, we brought all our equipment, including samples of media, to the base laboratory in Boston, and repeated our experiments, using the Labrador field equipment, on a group of college students. The results of these cultures were negative.

In a second experiment, we made nasopharyngeal washings on seven persons in the usual way. One-half of each washing was filtered by means of the Labrador hand pump and one-half by the Chapman water pump. The cultures of the filtrates from the hand pump were all negative; the cultures of filtrates from the Chapman pump were all positive. The two pumps were tested by the mercury manometer, and it was found that the hand pump produced a negative pressure of not more than 30 cm.,

whereas, with the Chapman pump, we were able to secure a negative pressure of at least 50 cm.

These results indicate that the filter-passing anaerobes commonly encountered in the nasopharynx cannot be filtered through a Berkefeld V filter unless considerable negative pressure is used (more than 30 cm. of mercury). This fact may explain the negative results of many observers who have attempted to cultivate these organisms. It might be added that under the conditions stated above none of the well-known aerobic flora pass through the filter, with rare exceptions (perhaps once in twenty trials).

In continuing the study in the West Indies, we overcame the filtration difficulties by substituting a Geryk pump (A. H. Thomas No. 1020). This pump, operated by hand, was found to create almost perfect vacuum as measured by a crude mercury manometer, and was used throughout the study in St. John.

RESULTS OF CULTURES TAKEN DURING AN EPIDEMIC OF INFLUENZA IN BOSTON

While we were at the base laboratory in Boston, a small epidemic of influenza occurred, and attempts were made to cultivate the filter-passing anaerobic nasopharyngeal flora of some of the typical cases.

The washings of this series were from fifteen normal persons and nine persons with epidemic influenza. Filter-passing anaerobic organisms were isolated from ten of the fifteen normal persons and from one of the nine influenza cases. Group IIIa organisms were recovered from all cultures showing growth, and were the only organisms found in eight of the ten normal persons and in the single positive culture from the influenza cases. One of the ten positive cultures from the normal persons also showed the presence of group II organisms, and another of the normal persons had group III organisms.

We cannot explain the negative findings in the cultures from the cases of influenza.

STUDIES IN ST. JOHN, VIRGIN ISLANDS, WEST INDIES

Nasopharyngeal washings. There were thirty-four nasopharyngeal washings in this series, sixteen from normal persons and

TABLE 3

Summary of findings in persons examined for filter-passing anaerobes in the naso-pharynx—St. John, United States Virgin Islands (West Indies)

AGE	CLINICAL CONDITION	BACTERIOLOGICAL FINDINGS (OLITSKY'S CLASSIFICATION)	REMARKS
14	Normal	II, Parvulus?	
14	Normal	IIIa, II	Shows 2 to 3 micra by 1 micron rods in plaque-like colonies
15	Normal	IIIa	
14	Normal	III, IIIa	These group III colonies show as "ground-glass" streaks
14	Normal	III or IIIa, III	Diplococcus, Parvulus-like
14	Normal	III	Short rods, not group IIIa
14	Normal	Growth, but unidentified	Three types
11	Normal	II, III	
13	Normal	III, IIIa	One type unidentified
20	Normal	II	Recovered from broth; solid medium contaminated
32	Normal	Unidentified	One type; diphtheroids also present
36	Normal	II? III	One type unidentified, resembles group II
14	Normal	I, III, IIIa	
19	Normal	III, IIIa, Ia	
35	Normal	IIIa, Ia, II?	
36	Normal	Ia, IIIa, II?	
13	Chronic cold	III, II?	
14	Cold	III? II or *B. pneumosintes*	
14	Chronic cold	II, IIIa?	
10	Cold, 1st day. Sore throat	II, III, IIIa	
14	Cold, 5 days	II? III	
14	Cold, 2 days	II, IIIa	
10	Cold	II	
11	Cold, 3 days	III, IIIa	
22	Acute cold, 2nd day	IIIa, III?, II?	
24	Acute cold, 2nd day	IIIa, II	
24	Cold, 8th day	II, IIIa, III?	
23	Cold, 7th day	IIIa	
37	Cold, 5th day	III?	
14	Cold, 1st day	II, IIIa, III?	
31	Cold, 1st day	I, II, III	
37	Sinusitis	III	
17	Cold, 1st day	I, III, IIIa	
23	Cold, 1st day	I, IIIa, II?	

eighteen from persons with common colds.   In every experiment, filter-passing anaerobic organisms were cultivated (see table 3).

B. pneumosintes was not found in any of these cultures, but a pneumosintes-like organism was found in one of the persons with a common cold.   Representatives of all the groups of organisms

TABLE 4

Summary of findings in persons examined for filter-passing anaerobes in the nasopharynx (nasopharyngeal swabs)—St. John, United States Virgin Islands (West Indies)

AGE	CLINICAL CONDITION	BACTERIOLOGICAL FINDINGS (OLITSKY'S CLASSIFICATION)	REMARKS
35	Normal	III, IIIa	
21	Normal	IIIa, III, II or B. pneumosintes	
37	Normal	Ia, IIIa	
35	Normal	Growth, but not identified	
21	Normal	Negative	
37	Normal	Growth, but not identified	
32	Normal	Negative	
23	Normal	IIIa	Three types: I?, Ia? III?
35	Normal	IIIa	One unidentified, not Ia
21	Normal	III	One type very short rods, probably diphtheroids
37	Normal	IIIa	
40	Normal	Growth, but unidentified	
9	Cold, one day	III, IIIa, II?	One type pneumosintes-like
48	Acute cold, 1st day	IIIa, B. pneumosintes	Agglutination not done
32	Acute cold, 1st day	II, III, IIIa	

in Olitsky's classification were isolated; five of the sixteen washings from normal persons and nine of the eighteen cultures from persons with colds yielded types that did not fit into Olitsky's classification.   In table 3 these strains are marked questionable or unidentified.

Nasopharyngeal swabs. Cultures were made from material which was secured by means of a large sterile cotton swab that was applied directly to the posterior nasopharynx. The swab was whipped in hormone broth and the broth was then filtered and cultured in the same manner as the nasopharyngeal washing. This method was used for several reasons; first, in order to get specimens from the nasopharynx only, thus eliminating organisms from the nose, throat, and mouth; second, to compare the findings of such work with those of the nasopharyngeal washings; third, individuals would submit more readily to nasopharyngeal swabbings than to washings.

Fifteen examinations were made on twelve normal persons and on three with acute colds. Positive results were secured in thirteen instances, the two negative cultures being from normal persons. One of two cultures from persons with acute colds taken during the first day of illness yielded *B. pneumosintes* and the other a pneumosintes-like organism. Diagnosis was made from cultural and morphological characteristics; agglutination tests were not made. Other types of filter-passing anaerobes found were similar to those of the previous series (see table 4).

Mouth washings. In order to determine the prevalence of this group of organisms in the mouth cavity, we washed out the mouths of a group of five persons with hormone broth, filtered the broth, and made cultures in the usual way. Two of the persons were examined once and each of the other three twice, at intervals of six weeks. Positive results were obtained from each examination. The organisms were similar to those of Olitsky's classification. Type I was found once, type III once and IIIa four times. Six additional types were isolated that did not fit definitely into Olitsky's classification, either because of colony formation or morphology. *B. pneumosintes* was not found in any of the cultures.

SUMMARY

Gram-negative anaerobic filter-passing organisms have been recovered in 92 of 114 examinations of individuals in widely separated communities and under the handicaps of field labora-

tory conditions. Positive cultures were obtained from normal persons, from patients with common colds, during the acute and later stages of the disease, and from persons with tracheitis, influenza, and dengue. The strains which we have cultivated are prevalent in normal throats, as well as in the nasopharynx of persons suffering from colds. Recoveries varied from 54 to 100 per cent, the highest percentage being obtained from examinations conducted in the tropics.

The types of organism isolated were similar to those found elsewhere by Olitsky and Gates, Olitsky and McCartney, Dochez, Noble, and others. Other types were isolated which could not be classified according to Olitsky's classification.

Bacterium pneumosintes was isolated from a case of acute nasopharyngitis, and pneumosintes-like organisms were present in ten other cultures—three from normal persons, five from persons with colds, and two from persons with influenza.

Some changes in technique, such as the use of various vacuum pumps and various methods of heating the catalytic units for anaerobic jars, were instituted to suit local conditions.

Blood-agar pour plates have proved to be superior to blood-agar surface plates, and hormone blood broth more satisfactory than Smith-Noguchi medium, under field conditions.

It has been established that the most satisfactory results are obtained when the washings are taken in or near the laboratory and when a negative pressure of 50 cm. of mercury is obtained in filtering the nasopharyngeal washing through a Berkefeld (V) filter.

The two series of examinations showing recovery of filter-passing anaerobes by means of nasopharyngeal swabs and mouth washings are too small to allow us to draw conclusions. The results indicate the presence of this group of organisms in material from the mouth as well as in the nose and nasopharynx.

Previous investigators have pointed out the lack of constancy in morphology and colony formation among this group of organisms. We have noted this characteristic throughout our studies, more particularly during the study in St. John, where we were unable to classify many of the strains in accordance with Olitsky's classification.

REFERENCES

BRANHAM, S. E., AND HALL, I. C.   1921   Jour. Infect. Dis., 28, 143.

BURKY, E. L., AND SMILLIE, W. G.   1929   Nasopharyngeal flora in health and during respiratory disease in an isolated tropical island (St. John, Virgin Islands).   Jour. Exper. Med., 50, 643.

DETWILER, H. K., AND HODGE, W. R.   1924   Jour. Exper. Med., 39, 43.

FISK, E., AND BURKY, E. L.   1925   Jour. Infect. Dis., 30, 128.

GARROD, L. P.   1928   Brit. Jour. Exper. Path., 9, 155.

GORDON, M. H.   1922   Jour. Roy. Army Med. Corps, 39, 1.

LEVINTHAL, W.   1928   Centralbl. f. Bakt., 106, 195.

LISTER, SIR S.   1922   South African Med. Rec., 20, 434.

LOEWE, L., AND ZEMAN, F. D.   1921   Jour. Amer. Med. Assoc., 76, 986.

McINTOSH, JAMES   1922   Med. Res. Council of Great Britain, Privy Council Special Report Series No. 63.

MILAM, D. F., AND SMILLIE, W. G.   1931   Jour. Exper. Med., 53, 733.

MILLS, K. C., SHIBLEY, G. S., AND DOCHEZ, A. R.   1928   Jour. Exper. Med., 47, 193.

NOBLE, W. C., JR., AND BRAINARD, D. H.   1928   Jour. Prev. Med., 2, 313.

OLITSKY, P. K., AND GATES, F. L.   1920   Jour. Amer. Med. Assoc., 74, 1497.

OLITSKY, P. K., AND GATES, F. L.   1922   Jour. Exper. Med., 36, 501.

OLITSKY, P. K., AND McCARTNEY, J. E.   1923   Jour. Exper. Med., 38, 427.

TRAUT, F E., AND HERROLD, R. D.   1930   Arch. Int. Med., 45, 412.

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AGGRESSINS

AN OUTLINE OF THE DEVELOPMENT OF THE THEORY AND NOTES ON THE USE OF THESE PRODUCTS[1]

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Aggressins may be defined as substances secreted by certain organisms under favorable conditions of growth, which have the property of inhibiting phagocytosis by a specific action on the leucocytes and reticulo-endothelial system. They may, therefore, facilitate the rapid development of normally sublethal infections of disease-producing organisms, resulting in death.

The introduction of aggressins into the body causes the production of specific anti-aggressins. An anti-aggressive serum acts specifically against the aggressin and does not destroy the disease-producing organism. It, however, allows the phagocytes to exert their normal powers of destroying the organisms.

Aggressins are non-toxic products of bacterial metabolism, thought to be secreted by the bacteria during the period of their greatest activity. It is impossible to state definitely whether these substances are secreted products or endo-products liberated only on the death and lysis of the bacterial cell. In either case they are absolutely non-toxic substances and to produce their typical action of enhancing virulence must be associated with a certain minimum number of living bacterial cells.

Aggressive substances were noted by Salmon and Smith (1884), who state "that the germs of such maladies are only able to multiply in the body of the individual attacked, because of a poisonous principle or substance which is produced during the

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multiplication of the germs." Bouchard (1892) is quoted by Zinsser (1923) as subscribing to such a theory. As early as 1892 he found that certain products of virulent cultures increased the invasive powers of the germs. Pasteur (1888) and Kruse (1893) also mention such substances.

BAIL'S NATURAL AGGRESSIN THEORY

Bail (1905 a) coined the term aggressin to explain the phenomenon observed by Koch that tubercular guinea pigs succumb rapidly when injected with tuberculin. He found that filtered peritoneal exudates from tubercular guinea pigs were especially active. Anthrax exudates were shown to be aggressive in action (Bail 1904).

Bail (1905 b) considered that aggressins had seven special properties. (1) They are secreted by the organism in the animal body during the course of a disease. (2) Sublethal doses of bacilli may become lethal when the specific aggressin is injected with them. (3) Lethal doses of bacilli which ordinarily would cause a slow infection only, cause a rapid and severe infection if aggressin is added. (4) The addition of aggressin neutralizes the bacteria-destroying power of immune serum in the peritoneal cavity of a guinea pig. (5) The injection of aggressin alone produces immunity. (6) The specific action of aggressins is on the leucocytes, preventing phagocytosis. (7) Anti-aggressive sera (Bail (1910)) are distinct from antibacterial sera and act specifically on the products secreted by the pathogenic organisms, thus allowing phagocytosis to take place.

Bail (1905 c) divides bacteria into three classes: (1) true parasitic, (2) half-parasitic, and (3) saprophytic according as to whether they produce, (1) abundant aggressin, (2) small amounts of aggressin, or (3) no aggressin. In the case of half-parasites, such as the cholera vibrio, passage through guinea pigs does not transform them into true parasites which act in minimum dosage. Half-parasites always require a certain minimum number of organisms before aggressive action can take place. In 1909, Bail (1910) found that passage of a virus through guinea pigs did not increase the toxicity of a culture but only its aggressiveness.

Bail used aggressins to produce immunity against anthrax (1904), typhoid (1905), typhus and cholera (1905 d), and Weil used aggressins in fowl cholera (1905).

Bail's theory of the formation of true aggressins by an interaction of the body and the germ has been elaborated by other workers, notably his co-worker Weil (1905), who showed that fowl-cholera aggressin is (1) non-toxic, (2) highly aggressive, while (3) organ extracts of infected guinea pigs are non-aggressive. The aggressin either works in direct combination with the organism or paralyses the normal anti-bacterial reactions. Later, Weil and Braun (1909) elaborate on the specific anti-aggressive action of anti-aggressive sera.

Bandi (1906) found that cholera vibrios produce a strong aggressin. He caims priority for this discovery, basing his claim on his publications with Terni in 1899 and 1900. Bruschettini (1908) produced aggressins by growing organisms enclosed in a collodion sac in the peritoneal cavity of animals. Burgess and Hosch (1909) increased the leucocytic invasion of the peritoneum by the use of aleurone, thus increasing the subsequent development of aggressin.

Zade (1909) found that passage of pneumococci through animals increased the resistance of these organisms to phagocytosis and that their aggressivity was also increased.

Schobl (1910) and Haslam and Franklin (Schoenleber, Haslam and Franklin 1917) used aggressive substances for the immunization of animals against blackleg. Matsumota (1926) and Salsbery (1926) used anthrax aggressins for immunization. Hruska (1926) found that Besredka's cuti-vaccination against anthrax could be best performed by the use of sterile edema fluids.

THE ENDOTOXIN OR CULTURAL THEORY OF AGGRESSINS

Wasserman and Citron (1907) and Citron (1906 a, b, c,) found that aggressins are produced in culture media. They considered these substances essentially endotoxins and possessing the following properties: (1) They stimulate virulence. (2) They produce immunity by the stimulation of anti-bodies.

(3) They cause the production of anti-aggressive sera which can be used for the passive immunization of other animals.

Citron (1906) used bacterial extracts or artificial aggressins to produce immunity in hog cholera. Citron and Putz (1907) immunized against fowl cholera and hemorrhagic septicemia of swine and wild animals by means of cultural aggressins.

Doerr (1906) showed that bacterial extracts have properties identical to those of the so-called aggressins. Levy and Fornet (1906) found that non-toxic filtrates of B. paratyphi are highly aggressive. These filtrates produce reactions identical with tissue aggressins. Levy and Graustrom-Woskoboinikow (1907) show that filtrates of B. typhi, B. paratyphi, and B. pyocyaneous are all non-toxic but highly aggressive.

Pane and Lotti (1907) extracted dysentery bacilli at 60° and noted that the extracts were highly aggressive. The use of these extracts caused guinea pigs to succumb to 1/1000 M.L.D. This action was found to be specific and quantitative. This aggressin was non-toxic but inhibited phagocytosis.

Roux and Chamberland (1887) found that black-leg edema fluids and fluid cultures freed from bacterial cells protected guinea pigs against infection. These products were strictly specific. Immunization by means of cultures and edema fluids could be used to differentiate Clostridium chauvei from Clostridium septicus. Nitta (1918) used blackleg culture filtrates in Japan from 1911. Eichorn (1917) introduced the Japanese method to the United States in 1917. Goss and Scott (1917) at the Kansas Agricultural Experiment Station developed an efficient blackleg filtrate.

Sauerbeck (1907) states that bacterial extract aggressins and tissue aggressins are both endotoxins liberated on the destruction of the organisms. He found that small doses were aggressive and large doses toxic and considers these substances to be weak toxins.

Scott (1927) demonstrated that blackleg tissue aggressin is identical with blackleg cultural filtrate in all respects.

Zschokke (1927) shows that blackleg aggressins are specific, both immunologically and aggressively, and that sublethal doses

of blackleg organisms are not activated by filtrates of *Clostridium Novyi, Clostridium septicus,* or *Clostridium Welchii* but are readily activated by filtrates of blackleg cultures.

Theoretical and chemical studies of these substances were made by Balsi (1907) who found that dialysis of coli aggressin yielded two substances, a non-toxic aggressive albuminous substance and a toxic globulin portion. The aggressive albumin was shown to be highly antigenic. He also showed (1908) that the albuminous substance inhibits phagocytosis and that it is neutralized by fresh serum (opsonin) or by immune serum.

Ingravelle (1910) and Sampietro (1913) confirmed Balsi's work with coli aggressin using typhoid aggressins. The albuminous portion of this aggressin was found to be aggressive and non-toxic. Levi and Vida (1908) failed to find any difference between the albuminous and globulin portions of aggressin.

Bruschettini (1907) noted that pneumococcic aggressin contains a specific anti-body which is not destroyed by heating to 56° C. It is, however, destroyed by heating for half an hour at 70° to 90°, C. this being considered as indicating that aggressins are not simple bacterial extracts.

Pirquet and Schick (1905) considered the phenomenon of Koch, the basis of Bail's theory of aggressins, as an anaphylatic phenomenon, and therefore, held Bail's theory of aggressins unnecessary.

Salus (1905), in using typhoid and colon aggressins, found that these substances were non-specific and therefore could not be used to separate the two organisms.

Scarano (1907) noted that the virulence of typhoid organisms depends upon their aggressivity. Scott (1925) demonstrated that aggressivity has no direct relationship to virulence in the case of blackleg, virulence depending on a factor found within the living active cells. This substance or property was named "lethal" substance.

Bail (1905 a) finds that heating aggressive tubercular exudates to 60°C. increases their activity. Scott (1925) reports that

heating blackleg filtrates and aggressins to 60°C. for one hour increases the aggressivity of both products. Zschokke (1927), however, finds that blackleg cultural aggressins lose a part or all their potency by heating to 60°C. Some commercial producers heat all their blackleg aggressins to 60°C. in the process of manufacture.

Waele (1907) found that typhoid aggressin can be separated into a thermostable aggressin and a thermolabile toxic portion. The thermolabile aggressin is similar to the bacterial extracts of Wasserman and Citron.

Trincas (1909) showed that anthrax aggressin produced from pleural exudates is dialysable into a globulin and an albuminous portion, the later being aggressive and antigenic. He considered the toxicity of the filtrate to be due to endo-toxins.

Capone (1921) suggested that: (1) Bacterial extracts exercise antibacteriolytical action *in vitro* identical to that produced by exudates. (2) Natural and artificial aggressins hinder bacteriolysis *in vitro* by altering the complement. (3) Aggressin does not exert any demonstrable action on bacteriotropic sera. (4) Aggressins annul or reduce to a minimum the phagocytic powers of leucocytes.

Zinsser and Dwyer (1913) showed that anaphylotoxin prepared by the centrifugalization of *B. typhi* mixed with guinea pig complement is aggressive in action. The aggressivity of this anaphylatoxin is non-specific.

Goss and Scott (1918) and Scott (1923) measured the potency of blackleg aggressins by titrating them against washed cells of blackleg cultures or anti-blackleg serum. In the washed culture test, one aggressive unit of aggressin is the amount of aggressive substance which will activate one minimal dose of washed cells to produce typical lesions and death in a guinea pig. In the neutralization test, the aggressive unit is the amount of aggressive substance which will stimulate 1 M.L.D. of blackleg culture to kill a guinea pig previously immunized by the injection of two units of anti-blackleg serum against 2 M.L.D. of culture. The doses usually used in the neutralization tests are 15 units of anti-blackleg serum followed in fifteen hours by 2 M.L.D. of

blackleg culture mixed with graduated doses of filtrate. The aggressive substance is titrated against the 13 units of serum which are not already neutralized by the injection of 2 M.L.D. of culture. A comparison of these two tests, Scott (1925), showed that the amount of aggressin required to stimulate a minimal amount of washed cells was the same as that required to neutralize one unit of anti-blackleg serum.

Singer (1925) found that anthrax aggressin does not inhibit phagocytosis but prevents the destruction of the ingested organisms, thus causing their rapid ejection, thereby allowing them to multiply. The specific action of aggressin is thought to be against the reticulo-endothelial system.

Normally the anthrax bacilli are rapidly removed from the circulation. If aggressin is injected, septicemia occurs very rapidly due to a complete paralysis of the histocytes. The injection of aggressin twenty-four hours before the injection of the organisms does not prevent the disappearance of the organisms from the blood stream but causes a rapid septicemia to follow.

Scott (1925) showed that the virulence of blackleg cultures did not depend on their aggressivity. Avirulent strains of *Clostridium chauvei* were shown to produce as much aggressive substance as virulent strains. Virulence was seen to be due to a "lethal substance" found in the living bacterial cells. Virulent cultures of *Clostridium chauvei* were said to consist of (1) living bacterial cells, (2) a so-called "lethal substance" found in these cells, and (3) aggressin produced by the living bacterial cells during their development.

Scott (1930) showed that ammonium dihydrogen phosphate and potassium bicarbonate increased the growth of blackleg cultures and also increased the production of aggressins. Ferric salts increased the production of aggressins and the virulence of blackleg cultures, but did not increase the amount of growth. Potassium citrate increased the aggressive action but did not increase the virulence or amount of growth. From these experiments it seems that aggressins are bacterial products which are produced by rapidly growing cultures. The production of these

substances can also be stimulated by direct action on the bacterial cells which causes an increase of aggressive substance without causing an increase in the number of bacterial cells.

Eastwood (1927) points out the mistakes in theory and in practice which have followed the use of the term "aggressin." He considers that the use of this nomenclature, which is based on that of human warfare, has a tendency to endow bacteria with properties of self-determination which they obviously do not possess. A better understanding of the problem of the interaction of organism and host would follow, if aggressins were regarded as merely one of many bacterial haptenes, and the term "aggressin" dropped.

IMPEDINS

Torikata (1917), referred to by Torikata and Fujimoto (1927), found that boiled filtrates of bacterial cultures gave higher precipitin reactions than the corresponding unheated filtrates. Torikata explained this phenomenon by suggesting the presence of an inhibiting substance, "impedin," in the unheated filtrate. This substance or energy called impedin he considers is developed during the development of the organism in culture media. Impedin prevents the union of the antigen with the cellular receptors and so prevents or greatly decreases the production of anti-bodies. Impedin is much more thermolabile than the antigenic complex so that boiled antigenic solutions or suspensions produce greater antigenic action in spite of the reduction in the actual amount of antigenic substance. The removal of the inhibiting substance more than compensates for the loss of antigenic substance. Torikata uses the term Koktoantigen to describe antigens which have been boiled. Nakagawa (1925) used Koktoantigens in the production of an antivariola serum with which he attempted to determine the potency of variola lymph.

Suguro (1925), working with staphylococci, found that the toxicity of bacterial emulsions depends on the dose used and that this toxicity determines a progressively increasing hyperleucocytosis. Unheated culture filtrates added to the bacterial

emulsion increase the toxicity, while boiled filtrates reduce the toxicity but increase the antigenicity as determined by phagocytosis. He determines the degree of phagocytosis by adding the number of phagocytes containing ingested cocci to the total number of ingested organisms. Suguro in this series of articles states that the total bacterial antigen or protein-lipoid complex consists of three parts: Toxophore, Haptophore, and Impedin. The toxophore group is very easily destroyed by heat, the haptophore group is relatively thermostable, and the impedin substance or energy is more stable than the toxophore but less stable than the haptophore group.

This author considers that impedins have an associative toxic action in that they prevent the development of anti-bodies and so allow the toxins free play. Bacterial cultures or bacterial filtrates containing impedins will, therefore, have a toxic action when added to bacterial toxins and aggressive action when added to living bacterial emulsions.

Torikata and Fujimoto (1927) show that boiled emulsions of dysentery organisms of the Shiga strain are non-toxic but highly antigenic. In a discussion of the numerous articles on impedins published in Japan they cite the work of Suzuki in Formosa on the use of Koktoantigen in the prevention of typhoid. These workers, (Torikata and Fujimogo (1928)) show the value of Koktoantigens in precipitation and complement fixation tests. They find that unheated cholera antigens may show non-specific complement fixation reactions in the Wasserman test but that boiled cholera antigens show only specific complement fixation reactions.

Torikata and Takamatsu (1929) in a series of articles find that the addition of boiled filtrates of colon cultures increases the production of agglutinins by colon vaccines, while filtrates of staphylococci also have the same action to nearly the same degree. From these experiments they consider that impedins are not species specific.

Fujimoto (1929) studies the action of Koktoantigens or boiled filtrates on the production of agglutinins by cholera vibrios. He also discusses the general application and theory of impedins.

VIRULINS

In a discussion of aggressins it is necessary to mention briefly another similar bacterial product or substance. Rosenow (1907) found that avirulent cultures of pneumococci could be made virulent by the addition of saline extracts of virulent cultures. The substance obtained from the virulent organisms is known as virulin.

In the production of immunity against disease and in the production of specific anti-bodies it is important to consider the import of the individual bacterial products, energies, or substances—aggressins, impedins, anaphylatoxins, virulins, lethal substances, as well as the perhaps better-understood substances, such as toxins, agglutinins, precipitins, etc.

The action of heat and drugs on immunizing agents is also important. It has been suggested that formaldehyde acts on toxins and aggressins in a definite manner and produces a more actively antigenic substance. Heat increases antigenicity, if it is high enough and acts long enough to destroy the impedin. It may also specifically increase the aggressivity or potency of aggressin substance. The culture medium used to produce antigenic emulsions or products may have a great effect on the product. Carbohydrates, salts, and other constituents may be of great importance.

The correlation of virulence or toxicity with antigenicity may be accidental rather than causal as often considered. This is suggested by the impedin work and also by experiments on aggressins produced by virulent and avirulent cultures.

AGGRESSINS IN VETERINARY PRACTICE

In 1915 Franklin and Haslam (Schoenleber *et al.* (1917)) of the Veterinary Department of the Kansas Agricultural Experiment Station developed blackleg tissue aggressin to a point where in field practice it proved highly successful.

Nitta (1918) used blackleg filtrates in Japan. Eichorn (1917) introduced this method to the United States. Goss and Scott (1917) produced a filtrate in Kansas, Graub and Zschokke

(1920) in Switzerland, Manninger (1924) in Austria, Zeller (1924) in Germany, Allen and Bosworth (1924) in England, Viljoen and Shewber (1926) in South Africa and many others. In all these countries excellent results have followed the use of these cultural filtrate aggressins.

The action of formaldehyde on tetanus toxins was studied by von Eisler and Lowenstein (1911) and von Eisler (1915). These workers used dilute concentrations of this disinfectant and also studied the effects of formaldehyde on blood serum and the agglutinins produced in immune sera (1912).

Ramon (1922) used formalin in the production of diphtheria toxoids which he called anatoxins, Descomby (1924) on tetanus anatoxins, while the work of Leclainche and Vallee (1925), and McEwen (1926) on blackleg anacultures and formalized filtrates has led to the extensive use of formolized blackleg cultures in Europe by Graub and others. Scott (1930 b) showed that formaldehyde acted directly and quantitatively on the aggressive substance of blackleg filtrates and tissue aggressins.

In 1922 Gochenour (1924) developed an aggressin for the immunization of animals against hemorrhagic septicemia. Recently Salsbery (1926) has developed an anthrax aggressin. Schilling (1929) finds that filtered anthrax edema fluids and filtered anthrax cultures, grown in media containing blood, milk or sterile tissue, develop considerable immunity in rabbits but none of these products have any aggressive or toxic actions. He (1927) also finds that abortus exudates have no aggressive action. This abortus product does not produce any immunity.

These three products, blackleg aggressin—natural or artificial, hemorrhagic septicemia aggressin, and anthrax aggressin, are fluid products containing substances produced by the growth of the specific organism in favorable media. They are non-toxic even in large doses and they produce immunity against the specific organism used to produce them. The blackleg products, blackleg filtrate, cultural or artificial aggressin, and natural or tissue aggressin, are the most efficient. The hemorrhagic septicemia aggressin is probably the least efficient, due to the nature of the disease. Losses from so-called shipping fever are nearly

twice as great among cattle vaccinated with hemorrhagic septi-cemia aggressins, vaccines, or bacterins at the stock yards or on arrival at the farm as among untreated cattle.

Aggressins, vaccines and bacterins produce a negative phase or period of increased susceptibility, during which the animals are highly susceptible to the disease. In the use of aggressins, it must be remembered that these products are aggressive and that they will stimulate non-lethal doses of the organism to cause disease and death.

Blackleg aggressins, filtrates and bacterins have produced aggressive action when used in infected herds. Losses have also been reported from the use of hemorrhagic septicemia aggressins in herds of sheep and flocks of chickens which were injected with this product during an acute attack of disease.

SUMMARY

1. Aggressins are substances produced by active bacterial cells growing under favorable conditions, whether in susceptible animals or in culture media.

2. Aggressins are non-toxic and they are specific.

3. Aggressins stimulate small doses of the corresponding or-ganism to produce acute disease and death.

4. Aggressins are thermostable.

5. Aggressins are increased in potency by the addition of formaldehyde.

6. The specific action of aggressins is to inhibit the phago-cytic mechanism, especially the reticulo-endothelial system.

7. According to Bail's theory, aggressins are produced by the bacteria only under conditions found in the diseased tissues.

8. Wasserman and Citron consider these substances to be bacterial extracts—non-toxic endotoxins or endo-products.

9. Aggressins may be considered as chemical substances pro-duced by bacterial cells in the animal body or in favorable cul-ture media. It is not clearly known whether aggressins are true bacterial secretions or whether they are endo-bacterial products liberated on the lysis of the cells.

10. Impedins are products of bacterial metabolism which de-

crease or prevent the combination of bacterial antigens with the cellular receptors of the host. Impedins decrease the production of precipitins, agglutinins, amboceptors, phagocytosis, and active immunity.

11. Impedins are thermolabile. They are destroyed by boiling at 100°C. for from ten minutes to one hour. Impedins are more stable than toxins but less thermostable than bacterial antigenic substances.

12. According to Torikata heating bacterial culture filtrates to 100° makes them valuable adjuvants to bacterial vaccines.

13. Impedins are not specific.

14. Impedins have toxic and aggressive actions in that they inhibit the production of antibodies.

15. Virulins are substances found in virulent bacterial organisms which, when added to avirulent organisms of the same species, render them virulent.

16. Aggressins should not be used in affected herds.

17. So-called natural aggressins and cultural aggressins or filtrates are identical as far as the antigenic substance is concerned.

REFERENCES

ALLEN, H. R., AND BOSWORTH, T. J. 1924 Vet. Jour., 80, 344.

BAIL, O. 1904 Cent. f. Bakt., Abt. 1, Orig., 37, 270–360.

BAIL, O. 1905 a Wein. Klin. Woch., 18, 211.

BAIL, O. 1905 b Munch. Med. Woch., 52, 1863–1935.

BAIL, O. 1905 c Arch. f. Hyg., 52, 302–328.

BAIL, O. 1905 d Wein. Klin. Woch., 18, 428.

BAIL, O. 1910 Folia Serol., 4, 129.

BANDI, I. 1906 Cent. f. Bakt., Abt. 1, Orig., 42, 448–450.

BLASI, D. DE 1907 Ann. d'Ig. Sper., 17, 253.

BLASI, D. DE 1908 Ann. d'Ig. Sper., 18, 79.

BOUCHARD 1892 Baillière et fils, Paris.

BRUSCHETTINI, A. 1907 Cent. f. Bakt., Abt. 1, Orig., 44, 441.

BRUSCHETTINI, A. 1908 Cent. f. Bakt., Abt. 1, Ref., 42, 417.

BURGERS AND HOSCH 1909 Zeit. f. Immun., Abt. 1, Orig., 2, 31–80.

CAPONE, G. 1921 Boll. d. Inst. Sieroterap. Milanese, 2, 17.

CITRON, J. 1905 Cent. f. Bakt., Abt. 1, Orig., 40, 153.

CITRON, J. 1906 a Cent. f. Bakt., Abt. 1, Orig., 41, 230–239.

CITRON, J. 1906 b Zeit. f. Hyg., 52, 238–262.

CITRON, J. 1906 c Zeit. f. Hyg., 53, 515.

CITRON, J. AND PUTZ, R. 1907 Zeit. f. Hyg., 56, 81; 145.

DESCOMBY, P. 1924 Compt. Rend. Soc. Biol., 91, 239.
DOERR, R. 1906 Cent f. Bakt., Abt. 1, Orig., 41, 497–593.
EASTWOOD, A. 1927 Jour. Hyg., 26, 235.
EISLER, M. VON, AND LOWENSTEIN, E. 1911 Cent. f. Bakt., Abt. 1, Orig., 61, 271–289.
EISLER, M. VON, AND LOWENSTEIN, E. 1912 Cent. f. Bakt., Abt. 1, Orig., 63, 261–281.
EISLER, M. VON 1915 Wien. Klin. Woch., 28, 1223.
EICHORN, A. 1917 Jour. Am. Vet. Med. Assoc., N. S., 6, 406.
FUJIMORI, K. 1929 Cent. f. Bakt., Abt. 1, Orig., 111, 68–83.
GOCHENOUR, W. S. 1924 Jour. Am. Vet. Med. Assoc., N. S., 18, 433.
GOSS, L. W. 1917 Kan. Agric. Expt. Sta. Circ. 59 (revised).
GOSS, L. W., AND SCOTT, J. P. 1918 Jour. Am. Vet. Med. Assoc., N. S., 7, 234.
GRAUB, E., AND ZSCHOKKE, W. 1920 Schweiz Arch. f. Tierhlk., 42, 52.
HRUSKA, CH. 1926 Ann. Inst. Past., 40, 710–712.
INGRAVALLE, A. 1910 Ann. de'Ig. Sper., 20, 483.
KRUSE, W. 1893 Beiträge Path. Anat. U. Allg. Path., 12, 333.
LEVI, M. DELLA VIDA 1908 Ann. de'Ig. Sper., 1, 91.
LEVY, E., AND FORNET, W. 1906 Deut. Med. Woch., 28, 1039.
LEVY, E., AND GRAUSTROM-WOSKOBOINOKOW 1907 Cent. f. Bakt., Abt. 1, Orig., 45, 360–365.
LECLAINCHE, H., AND VALLEE, E. 1925 Compt. Rend. Soc. Biol., 92, 1273.
McEWEN, A. D. 1926 Jour. Comp. Path. and Therap., 39, 253.
MANNINGER, R. 1924 Cent. f. Bakt., Abt. 1, Orig., 92, 418.
MATSUMOTO, T. 1924 Zeit. Immunfrsh. U. Expt. Therap., 40, 402–428.
NAKAGAWA, S. 1925 Zeitschr. f. Immunitatsforsch., 44, 300.
NITTA, N. 1918 Jour. Am. Vet. Med. Assoc., N. S., 6, 446.
PANE AND LOTTI 1907 Cent. f. Bakt., Abt. 1, Orig., 43, 718.
PASTEUR, L. 1888 Compt. Rend. Acad. Sci., 106, 320–322.
PIRQUET, C., AND SCHICK, B. 1905 Wein. Klin. Woch., 18, 431.
RAMON, G. 1922 Compt. Rend. Soc. Biol., 86, 813.
ROSENOW, E. C. 1907 Jour. Inf. Dis., 4, 285.
ROUX, E., AND CHAMBERLAND, CH. 1887 Ann. Inst. Past., 1, 561.
SALUS, G. 1905 Wein. Klin. Woch., 18, 660.
SALMON, D. E., AND SMITH, TH. 1884 Proc. Biol. Soc., 18, 29–33.
SALSBERY, C. E. 1926 Jour. Am. Vet. Med. Assoc., N. S., 23, 359–372.
SAMPEITRO, G. 1913 Ann. De'Ig. Sper., 23, 112.
SAUERBECK, E. 1907 Zeit. f. Hyg., 56, 81.
SCARANO, P. 1907 Riforma Med. 23, No. 3.
SCOTT, J. P. 1923 Jour. Am. Vet. Med. Assoc., N. S., 17, 67.
SCOTT, J. P. 1925 Jour. Bact., 10, 265–313.
SCOTT, J. P. 1927 Indian Vet. Jour., 3, 290.
SCOTT, J. P. 1930 a Jour. Am. Vet. Med. Assoc., N. S., 29, 525.
SCOTT, J. P. 1930 b Jour. Inf. Dis., 46, 460.
SCHULLING, S. J., AND BLEECKER W. L. 1927 Jour. Inf. Dis., 40, 469.
SCHILLING, S. J. 1929 Univ. Ark. Agric. Expt. Sta. Bull. 238.
SCHOBL, O. 1910 Cent. f. Bakt., Abt. 1, Orig., 56, 395–399.
SCHOENLEBER, F. S., HASLAM, T. P., AND FRANKLIN O. M. 1917 Kansas Agric. Expt. Sta. Circ. 59.

SINGER, E. 1925 Zeitschr. f. Immun., **45**, 12.

SUGURO, H. 1925 Zeitschr. f. Immunitatsforsch., **42**, 58–81.

SUYUKI, SHIMOMIYA, AND HIRATA 1924 Rpt. Hyg. Dept. Central Institute of Formosan Government, 1924, No. 1730.

TORIKATA, R., AND FUJIMOTO, A. 1927 Jour. Immun., **13**, 123–159.

TORIKATA, R., AND FUJIMORI, K. 1928 Zeitschr. f. Immunitatsforsch., **56**, 155–174.

TORIKATA, R., AND TAKAMATSU, I. 1929 Cent. f. Bakt., Abt. 1, Orig., **110**, 214–220; 220–227; 227–233.

TRINCAS, L. 1909 Ann. de'Ig. Sper., **19**, 19.

VILJOEN, P. R., AND SCHEUBER, J. R. 1926 Union of South Africa. Dept. Agr. Rpts. Dir. Vet. Ed. and Research, 11–12, pt. 1, 499.

WAELE, H. DE 1907 Cent. f. Bakt., Abt. 1, Orig., **44**, 360.

WASSERMAN, A., AND CITRON, J. 1907 Cent. f. Bakt., Abt. 1, Orig., **43**, 373–376.

WEIL, E. 1905 Arch. f. Hyg., **52**, 412–432.

WEIL, E. 1908 Arch. f. Hyg., **65**, 81–105.

WEIL, E., AND BRAUN, H. 1909 Folia Serol., **3**, 27.

ZADE, M. 1909 Zeit. f. Immun., **2**, 81–97.

ZELLER, H. 1924 Ber. Tier. Woch., **40**, 49.

ZINSSER, H., AND DWYER, J. L. 1913 Proc. Soc. Exper. Biol. and Med., **11**, 74.

ZINSSER 1923 Macmillan, 3rd Ed.

ZSCHOKKE, W. 1927 Schweiz. Arch. f. Tierhlk., **7**, 357.

THE EFFECT OF SODIUM RICINOLEATE ON THE GONOCOCCUS[1]

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Beginning with some interesting observations on the effect of surface tension depressants on the growth of bacteria in culture media Larson and his co-workers (Larson, Cantwell and Hartzell, (1919)) have published a number of papers on the action of sodium ricinoleate upon certain organisms and toxins. This soap was selected from a large group of fatty acid soaps investigated because it was the most effective, was readily soluble in water and could be used at a hydrogen ion concentration not far below that of ordinary culture media.

They reported that certain bacteria, such as pneumococci and streptococci, were rendered non-pathogenic (Larson, Cantwell and Hartzell (1919); Larson (1925–26); Larson and Eder (1926); Larson and Montank (1922–23); Larson (1928a) and the toxins of diphtheria, tetanus and scarlatina non-toxic (Larson and Nelson (1923–24); Larson, Evans and Nelson (1924–25); Larson and Eder (1926); Larson (1928a) by treatment with sodium ricinoleate, although the ability of the organisms to engender specific antibodies was not impaired.

They explain this "detoxifying" action on the hypothesis that the soap is adsorbed to the surface of the toxin molecules or molecular aggregates (Larson, Evans and Nelson (1924–25); Larson, Halvorson, Evans and Green (1925)).

Larson, Huenekens and Colby (1926) recommended ricinoleated toxin for the immunization of children, and reports of such a pro-

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cedure have already appeared (Colby (1926); Perkins and Megrail (1926)).

Kozlowski (1928a and b) found that mice could be successfully immunized against scarlatinal streptococci by vaccination with ricinoleated organisms and toxin. He doubted Larson's explanation, however, and suggested that the local necrotizing action of the soap on the tissues of the animal (which often caused ulceration) might delay the absorption of the antigen, an explanation which obviates the necessity of the adsorption hypothesis. In a reply to Kozlowski's paper, Larson (1928b) stated that ulceration produced by Kozlowski's ricinoleate was probably due to the presence of traces of barium which remain from the process of purification, as he had found that sodium ricinoleate so prepared always produced ulceration, whereas his later method (not described) yielded a preparation which was much less toxic. Larson's explanation was criticized by Kozlowski (1929), who reasserted his original contention that pure sodium ricinoleate is, itself, very toxic.

Netter, *et al.* (1927) were able to vaccinate rabbits with ricinoleated streptococci, but found no evidence that their sera conferred passive immunity upon mice.

McKinley and Larson report (1926–27) some experiments which suggest strongly, but not conclusively, that monkeys can be vaccinated against experimental poliomyelitis by means of ricinoleated virus.

Carmichael (1927) has found that sodium ricinoleate detoxifies rattlesnake venom.

Some observations on the effect of sodium ricinoleate on the turbidity of bacterial suspensions have recently been reported by Spencer (1930). Among a great number of species tested, the effect varied from complete clarification to actual increase in turbidity. The gonococcus was not studied.

Our interest in the effect of sodium ricinoleate on the gonococcus arose in connection with two lines of investigation on the biology of this organism. The first was a study of the influence on its growth of various ingredients in culture media, and the second was related to its toxicity for laboratory animals. No mention

could be found in the literature of the treatment of gonococci with soaps, so we proceeded to investigate the matter ourselves. It would be both useful and theoretically interesting to be able to "detoxify" the gonococcus without destroying its antigenic properties.

MATERIALS

Cultures. The strain of gonococcus employed in these experiments was isolated by us from the urethral pus of a patient suffering from typical acute gonorrheal urethritis. It had been under cultivation in our laboratory for many months.

Media. In all experiments the organisms were grown on the medium described by Miller and Castles (1930), which consists of a tryptic digest of egg white, glucose, agar and a mixture of salts. Except in experiment 1, in which slopes were used, the organisms were cultivated in ordinary flat-sided medicine bottles. As experience had shown that growth on this medium reaches its maximum in twelve to fifteen hours, the organisms were washed off at the end of that time with saline, strained through cheese cloth to remove particles of agar, centrifuged, washed in saline and centrifuged at high speed in weighed centrifuged tubes. After the supernatant fluid had drained off, the weight of the packed, wet organisms was determined. This was found to be the easiest satisfactory method of estimating the dosage of inocula.

The sodium ricinoleate[2] was dissolved in saline and autoclaved. Some preliminary experiments convinced us that autoclaving did not affect the toxicity of the solution.

EXPERIMENTAL

The effect of sodium ricinoleate on the growth of gonococcus on artificial media

Experiment 1. The ricinoleate was dissolved in saline, autoclaved and added to the melted agar in the dilutions indicated in table 1. It did not materially affect the pH, that is, it did not raise it beyond the limits within which the gonococcus will grow

[2] The authors desire to express their indebtedness to Eli Lilly and Company of Indianapolis for furnishing them with pure sodium ricinoleate.

luxuriantly on this medium. The cultures were examined for growth at the end of twelve and twenty-four hours.

Table 1 shows that growth was completely inhibited by sodium ricinoleate in a concentration of 1:20,000 and was scant in a concentration of 1:200,000.

TABLE 1

Growth of gonococcus on culture medium containing sodium ricinoleate

CONCENTRATION OF SODIUM RICINOLEATE	GROWTH
1:1,000	0
1:2,000	0
1:10,000	0
1:20,000	0
1:100,000	+
1:200,000	++
0	++++

TABLE 2

Comparison of the toxicity of ricinoleated and untreated gonococci in mice

The fractions indicate the number in each pair which died

CONCENTRATION OF SODIUM RICINOLEATE	APPROXIMATE WEIGHT OF GONOCOCCI						
	100 mgm.	50 mgm.	20 mgm.	10 mgm.	5 mgm.	1 mgm.	0 control
per cent							
1	2/2	1/1	2/2	2/2	2/2	2/2	1/2
0 1	2/2	2/2	1/2	2/2	1/2	0	0
0.01	2/2	2/2	1/2	1/2	1/2	0	0
0 control	2/2	2/2	1/2	1/2	0	0	

With one exception death, when it occurred, took place within twenty-four hours.

The series receiving concentration of sodium ricinoleate greater than 1 per cent have been deleted, because the mice all died within half an hour.

The effect of sodium ricinoleate on the killing power of gonococci for mice

Experiment 2. Series of mice were injected intraperitoneally with varying doses of gonococci suspended in varying dilutions of sodium ricinoleate. The dilutions were so made up that the quantity of each inoculum was 1 cc. About two hours elapsed

between the time the suspensions were made and the time the inoculations were made. The tests were run in duplicate.

The results, contained in table 2, show that treatment of gonococci with 1 per cent sodium ricinoleate considerably increased their lethal action for mice. (The M.L.D. was not determined as it fell below the limits of the experiment.) Treatment with 0.1 and 0.01 per cent had a similar, though less pronounced effect. One of the pair of mice receiving 1 per cent ricinoleate alone was killed, showing that the soap in that concentration is itself toxic.

This experiment was repeated three times, once employing a recently isolated strain, with results practically identical with those shown in table 2.

TABLE 3

Relative numbers of mice killed by gonococci treated with 0.1 per cent sodium ricinoleate and by untreated gonococci

	APPROXIMATE WEIGHT OF ORGANISMS			
	20 mgm	10 mgm	2 mgm.	0 control
Ricinoleated	10/10	4/10*	1/10	2/10
Untreated	4/10	1/10	0	

* These mice were given half of the dose of the same suspension given the mice represented by the preceding fraction; they therefore received only 0 5 cc. of 0.1 per cent ricinoleate, whereas all the rest received 1 cc.

Experiment 3. To determine whether the length of time or the temperature at which the gonococci were in contact with the ricinoleate might influence the results we had obtained, the following experiment was performed: Suspensions of gonococci, in varying dilutions were made up in 1 and in 0.1 per cent ricinoleate as in experiment 2, and injected immediately into mice. Control mice received corresponding doses of gonococci in saline, and other controls, sodium ricinoleate alone. A second set of mice was injected with the same suspensions and solutions after they had stood at room temperature for two hours. The liquids were then divided. One set was kept at room temperature; the other in the ice box. A third series of mice was injected at the end of eighteen hours, and a final series at the end of seventy-two hours with the

materials kept on ice.   The results in all four series were identical, within the limits of experimental error, and similar to those of experiment 2, showing that the action of sodium ricinoleate on gonococci was rapid and that the enhancement of their killing power was not affected by prolongation of treatment.

Experiment 4.   In the preceding experiments the number of mice inoculated with a given suspension was limited to two.   In spite of the uniformity of the results it was considered advisable to gain some notion of the factor of individual susceptibility by increasing this number to 10.   The method was the same as in experiment 2.   As will be seen in table 3, a larger number of mice were killed by gonococci treated with 0.1 per cent ricinoleate than by the corresponding dose of untreated organisms.

The effect of sodium ricinoleate on pneumococci and streptococci

Our findings were at such variance with the results reported by Larson and others of the "detoxifying" effect of ricinoleate that we suspected that the ricinoleate we were using might be at fault. We therefore repeated and confirmed his observations on pneumococci and scarlatinal streptococci.   Treatment of these organisms with 0.1 per cent sodium ricinoleate rendered them so nearly innocuous that mice withstood very many lethal doses (in the case of a virulent type I pneumococcus, 1 million).   The efficacy of our ricinoleate as regards its effect on these organisms was thereby established.

Gross and microscopic appearance of gonococci and pneumococci suspended in ricinoleate

Aliquot portions of gonococci were suspended in 1 and 0.1 per cent sodium ricinoleate (dissolved in saline) and in saline. Smears were made immediately, after one-quarter, one-half, three-quarters, one and two hours and again after standing overnight in the ice-box.   Each smear was made with a loopful of suspension and covered approximately the same area on the slide.   The smears were stained by Gram's method.   Macroscopically the suspensions became rapidly less turbid as compared with the saline control.   No turbidity standards were used, but it was our

impression that the clearing reached its maximum in about fifteen minutes.   Microscopic examination of the smears made immediately showed the ricinoleated organisms to be somewhat smaller and not quite so well stained as those in the saline control.   A small amount of amorphous material was present.   The other smears, taken in order, showed a progressive decrease in the number of formed organisms and in the intensity of their staining, and a progressive increase in the amount of amorphous material. The eighteen-hour suspension still showed some formed organisms of various sizes.   The series of smears of the saline suspensions showed practically no change throughout.

Suspensions of pneumococci type I were treated in the same way.   Macroscopically the ricinoleated suspensions showed rapid decrease in turbidity but never became water-clear.   Microscopic examination of the smears showed a progressive diminution in the number of formed organisms and among these, a progressive increase in the number of Gram-negatives.   The amorphous material, which increased progressively, was in all instances Gram-negative.

The antigenic properties of gonococci treated with sodium ricinoleate

Two series of eight rabbits each were immunized against ricinoleated gonococci.   The antigen for series I was treated with 1 per cent sodium ricinoleate, that for series II with 0.1 per cent. The ear veins in most instances became inflamed so that many of the injections had to be made intraperitoneally.   Aside from that, the animals stood the immunization very well.   The titers of agglutinating and complement-fixing antibodies of the sera in both series (tested against untreated antigens) fell within the limits of the titers engendered by untreated gonococci.

<div align="center">DISCUSSION</div>

Experiment 1 shows clearly that sodium ricinoleate inhibits the growth of gonococci in very low concentration (1 part in 20,000) on a solid medium which itself is quite satisfactory for that organism.   This finding places the gonococcus within the group of organisms (pneumococci, streptococci, etc.) described by Larson,

Cantwell and Hartzell as being extremely sensitive to the action of surface tension depressants.

The killing power of this organism for mice, on the other hand, is enhanced by treatment with sodium ricinoleate and in this respect it differs from pneumococci and streptococci. The explanation for this discrepancy becomes quite simple if one calls to mind the essential difference between the gonococcus and the pneumococcus or streptococcus in its method of killing mice. The latter organisms are able to multiply rapidly within the peritoneal cavity of the mouse; the gonococcus has no such capability; it kills its host only when it is injected in enormous numbers and because it contains toxic substances within its body. (We are leaving out of consideration the disputed question whether or not any of these substances may be a true toxin.)

Since sodium ricinoleate dissolved a large fraction of the gonococci, it seems reasonable to suppose that the absorption of their toxic constituents was facilitated. Added thereto was the somewhat toxic ricinoleate itself. This simple explanation seems to us to suffice to account for the enhancement of the killing power of gonococci treated with sodium ricinoleate.

The marked reduction in pathogenicity of pneumococcus and streptococcus accomplished by such treatment seems to us equally simple. It will be recalled that the greater portion of these organisms were also dissolved, and most of the rest so injured that they failed to retain the Gram stain. This means that the number of healthy organisms must be extremely diminished, and since their killing power depends essentially on their ability to reproduce themselves within the body of the host one would expect that the inoculum of ricinoleated organisms would have to be enormously increased before a minimum fatal dose would be attained.

SUMMARY

1. Sodium ricinoleate inhibited the growth of gonococcus on artificial media in very high dilutions.

2. Gonococci suspended in 0.1 and 1 per cent solutions of sodium ricinoleate were in large part dissolved (as are pneumococci and streptococci).

3. Such suspensions killed mice in smaller doses than suspensions of untreated gonococci. The same result was obtained with gonococci treated for as long as seventy-two hours with sodium ricinoleate.

4. Ricinoleated gonococci inoculated into rabbits engendered specific agglutinating and complement-fixing antibodies in as high titers as did untreated gonococci.

5. The difference between the effect of sodium ricinoleate on the killing power of the gonococcus and of the streptococcus and pneumococcus probably depends on the differences in the mechanism of pathogenicity.

CONCLUSIONS

1. Sodium ricinoleate does not "detoxify" gonococci but rather increases their lethal action for mice.

2. It does not destroy the antigenic proterties of gonococci.

REFERENCES

CARMICHAEL, EMMETT, B. 1927 Detoxification of rattlesnake venom by sodium ricinoleate. Jour. Pharmacol. and Exper. Therap., 31, 445.

COLBY, WOODARD 1926 The control of scarlet fever in institutions by active immunization with toxin detoxified with sodium ricinoleate. Jour. Amer. Med. Assoc., 87, 919.

KOZLOWSKI, ANTHONY 1928a A comparative study of the action of sodium ricinoleate upon bacteria. Jour. Bacteriol., 16, 203.

KOZLOWSKI, ANTHONY 1928b The effect of ricinoleated vaccine of the hemolytic streptococcus (scarlet fever) on animals. Jour. Immunol., 15, 115.

KOZLOWSKI, ANTHONY 1929 Ricinoleated vaccines. Jour. Immunol., 16, 357.

LARSON, W. P. 1925-26. A report on the preparation of pneumococcic antitoxin. Proc. Soc. Exper. Biol. and Med., 23, 297.

LARSON, W. P. 1928a The effect of the surface tension of the menstruum upon bacteria and toxins, Chapter XI, in "The Newer Knowledge of Bacteriology and Immunology" by Jordan and Falk, University of Chicago Press.

LARSON, W. P. 1928b Ricinoleated streptococcic vaccines. Jour. Immunol., 15, 299.

LARSON, W. P., CANTWELL, W. F., AND HARTZELL, T. B. 1919 The influence of the surface tension of the culture medium on the growth of bacteria. Jour. Infect. Dis., 25, 41.

LARSON, W. P., AND EDER, HOWARD 1926 Immunization against diphtheria with toxin detoxified with sodium ricinoleate. Jour. Amer. Med. Assoc., 86, 998.

LARSON, W. P., EVANS, R. D., AND NELSON, EDMOND 1924–25 The effect of sodium ricinoleate upon bacterial toxins, and the value of soap-toxin mixtures as antigens. Proc. Soc. Exper. Biol. and Med., **22**, 194.

LARSON, W. P., HALVORSON, H. O., EVANS, R. D., AND GREEN, R. G. 1925 The effect of surface tension depressants upon bacterial toxins. Colloid symposium Monograph, **3**, 152.

LARSON, W. P., HUENEKENS, E. J., AND COLBY, WOODARD 1926 Immunization against scarlet fever with toxin detoxified with sodium ricinoleate. Jour. Amer. Med. Assoc., **86**, 1000.

LARSON, W. P., AND MONTANK, IRWIN A. 1922–23 The effect of wetting on the pathogenicity and viability of tubercle bacillus. Proc. Soc. Exper. Biol. and Med., **20**, 229.

LARSON, W. P., AND NELSON, EDMOND 1923–24 The effect of surface tension of the medium upon bacterial toxins. Proc. Soc. Exper. Biol. and Med., **21**, 278.

McKINLEY, J. C., AND LARSON, W. P. 1926–27 Sodium ricinoleate and active immunity against experimental monkey poliomyelitis. Proc. Soc. Exper. Biol. and Med., **24**, 297.

MILLER, C. PHILLIP, JR., AND CASTLES, RUTH 1930 An egg white digest medium for the gonococcus. Proc. Soc. Exper. Biol. and Med., **28**, 123.

NETTER, ARNOLD, ANDRÉ, EMILE, CÉSARI, ET COTONI 1927 Action duriciléate de sodium sur les bactéries et les toxines. Comptes rendus Soc. de Biol., **96**, 184.

PERKINS, R. G., AND MEGRAIL, E. 1926 Use of sodium ricinoleate toxin (Larson) in immunization against scarlet fever. Ohio State Med. Jour., **22**, 599.

SPENCER, R. R. 1930 The visible effect of castor-oil soap on certain organisms. Pub. Health Repts., **45**, 1354.

STUDY OF BACTERIAL FLUORESCENCE IN VARIOUS MEDIA

I. INORGANIC SUBSTANCES NECESSARY FOR BACTERIAL FLUORESCENCE

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INTRODUCTION AND HISTORICAL

While working with fluorescing organisms it was found that certain lots of media failed to show the production of fluorescence when used for the growth of *B. fluorescens-liquefaciens*.[1] However, media of the same composition, when made with different lots of material, gave a good production of pigment. In looking up the literature on this subject it was found that conflicting statements appeared as to the constituents necessary for the production of fluorescence.

One of the first synthetic media used for the production of pigment with fluorescing organisms was proposed by Hueppe (1880). It contained a solution of ammonium tartrate, neutral potassium phosphate, magnesium sulphate, and calcium chloride.

Gessard (1892) studied the production of fluorescence with *B. pyocyaneus* in a medium very similar to that used by Hueppe. He found that phosphates were essential for pigment production, and that $\frac{1}{50000}$ part of phosphate could be detected in a medium by the production of fluorescence. Nägeli (1895) stated that calcium could be substituted for magnesium and that fluoresence could still be obtained. Thumm (1895), while working with *B. pyocyaneus*, studied fluorescence in a medium similar to that used by Gessard. For the organic constituent, he tried a number of

[1] This organism is now called *Pseudomonas fluorescens* by Bergey.

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organic compounds and concluded that, in addition to a source of organic nitrogen, a phosphate and magnesium sulphate must be present.   He disagreed with Nägeli's conclusion that calcium could be used in lieu of magnesium.

Lepierre (1895) studied a fluorescent bacillus which he isolated from water.   He found that the only common organic acid salts, which gave fluorescence when used with the proper inorganic constituents, were citric, succinic, oxy-glutamic, and glutaric acids.   He concluded that fluorescence depends, first, upon the dibasicity of the acid; second, upon the presence of at least two CH_2 groups in the molecule.

Jordan (1899) studied the production of fluorescence with six species of fluorescing bacteria.   He used ammonium salts of a number of organic acids with magnesium sulphate and sodium phosphate.   He found that the dibasicity of the acid and the presence of CH_2 groups were not essential as stated by Lepierre. Jordan concluded that a sulphate and a phosphate salt were the only inorganic constituents needed, and that, regardless of the base, equally good fluorescence was given.   In his words: "The nature of the base associated with the phosphorous and sulphur appears to be a matter of complete indifference.   Sodium, potassium, and magnesium salts give similar results in whatever way they are combined.   Even if ammonium phosphate and ammonium sulphate be used together, fluorescence appears, but it is somewhat less intense than in the presence of one of the bases mentioned above."

Jirou (1901) found that the simplest medium for production of fluorescence consisted of a mineral salt containing nitrogen, a hydrocarbon, and an alkaline or alkaline-earth phosphate. Ammonium citrate will suffice, he stated, if one adds ammonium carbonate and a phosphate.

Sullivan (1905) agreed with Jordan.   He concluded "that the fluorescent pigment could be formed whenever, in addition to asparagine, there was present in the solution both phosphates and sulphates, irrespective of the base."   He also agreed, in the main with Lepierre that the production of pigment was favored by the carboxyl and methylene groups.

Tanner (1918) studied one hundred strains of fluorescent bacteria isolated from water in Uschinsky's medium, Sullivan's medium, and Frankel's medium. In addition to other ingredients, the two media first mentioned contain magnesium sulphate and a phosphate. Frankel's medium contains sodium chloride, mono-calcium phosphate, ammonium lactate, and asparagine. According to Tanner all three of these media gave a good pigment production. It will be noted that Frankel's medium contains neither sulphates nor magnesium. One, therefore, might conclude that these elements were not essential for the production of fluorescence. However, some of the chemicals may have been impure.

As will be noted by the foregoing brief survey of the literature all investigators do not agree on the constituents especially the inorganic salts, which are necessary for the production of fluorescence. All of them, however, believe that some nitrogen containing organic compound, such as asparagine or ammonium succinate, is necessary as the organic constituent. Therefore, the organic compounds necessary for fluorescence will be considered settled: asparagine will be taken as the organic constituent in this research, and the constituents under investigation will be the inorganic salts.

METHODS AND MATERIALS

In all, ten different cultures of fluorescing organisms were used. Four of these were from standard collections and six were isolated from water supplies. All of these cultures were identical with, or closely allied to, *Pseudomonas fluorescens* (Flügge).

Great care was used in the selection of glassware and chemicals, owing to the fact that the least amount of impurity might vitiate the results. The glassware was thoroughly cleansed, rinsed in tap water, then with alcohol, and finally three times with distilled water. The chemicals used were the purest to be obtained on the market. In most cases analyzed chemicals were used. These were, however, tested for impurities. Over fifty samples were analyzed, and it was found that many of the samples contained far too great an amount of impurities, so that several recrystallizations were necessary in many cases.

All of the media were made up very carefully with water, redistilled from pyrex glass. A liter of this water gave no weighable residue. About 7 cc. of the nutrient solution were placed in each test tube and sterilized in steam for thirty minutes on three successive days.

Inoculations were made from forty-eight-hour broth cultures. In cases where the various sulphates were being tested for their effect on fluorescence, the broth contained no magnesium salts for fear that a trace of magnesium might be carried over into the medium tested. Likewise, in cases where some other salt was being tested for a given impurity, this ingredient was eliminated from the broth in which the stock cultures were grown.

EXPERIMENTAL

A number of preliminary experiments indicated that very good fluorescence was produced when asparagine, magnesium sulphate, and dipotassium hydrogen phosphate were used as a basis for the medium. Experiments were then conducted to see if this medium could be simplified. Media were made containing the following ingredients in varying amounts: (1) asparagine, (2) asparagine and a potassium phosphate, (3) asparagine and magnesium sulphate, (4) asparagine and magnesium chloride, (5) asparagine and the various alkali sulphates. None of the above media gave any fluorescence except in a few isolated tubes.

As noted in the historical review, Jordan and Sullivan state that sulphates and phosphates, regardless of the base, are the only inorganic elements necessary for the production of fluorescence. Therefore, this phase was next investigated. Media were prepared containing 0.2 per cent asparagine, with varying amounts of dipotassium phosphate and sulphates of sodium, potassium, and ammonium; and also sulphuric acid. Control media were also prepared from each of the above media with the addition of 0.01 per cent anhydrous magnesium chloride. Very little fluorescence was produced in any of the media without magnesium as may be seen by the results of one of the experiments given in table 1. Table 2 represents the same media plus 0.01 per cent magnesium chloride. The quantity of fluorescence is indicated by plus signs.

Four plus signs indicate the maximum.   A negative mark indicates no fluorescence.   Final readings were made at the end of four days.   In all of the experiments where no fluorescence was given, a satisfactory growth was, nevertheless, produced.   The lack of fluorescence, therefore, can not be charged to lack of growth.

The experiments with the other sulphates gave similar results to those recorded in tables 1 and 2.   These results seem to indi-

TABLE 1

Growth of fluorescing organisms in media containing asparagine, K_2SO_4, and K_2HPO_4

K_3SO_4	K_2HPO_4			
	0.001 per cent	0 01 per cent	0.1 per cent	0 2 per cent
per cent				
0 0001	—	—	—	—
0 001	—	—	+	—
0.01	—	—	—	—
0 1	—	+	—	+
0 2	+	++	+	+

TABLE 2

Growth of fluorescing organisms in media when 0.01 per cent $MgCl_2$ was added

K_2SO_4	K_2HPO_4			
	0 001 per cent	0 01 per cent	0 1 per cent	0 2 per cent
per cent				
0 0001	+	+	+	—
0 001	+	+++	++++	++
0 01	++	++++	++++	+++
0 1	++	++++	++++	+++
0.2	+	++	++	—

cate that fluorescence will not be produced equally well by any sulphate, save that of magnesium.   In some cases, the media containing the larger amounts of the sulphates gave a slight production of pigment.   It was thought that these might contain a small amount of magnesium as an impurity.   However, when 5 grams were analyzed no magnesium was detected.

A large number of C.P. analyzed sulphates were obtained. Five samples of each of the sulphates of sodium, potassium, and

ammonium were selected.   Analysis of each sample was made for magnesium, using 100 grams.   The samples of sodium sulphate contained magnesium sulphate varying from 0.003 to 0.0184 per cent, the potassium sulphates from 0.0039 to 0.0432 per cent, and the ammonium sulphates from none to 0.0014 per cent.

Analyses were also made on five samples each of the phosphates of sodium, potassium, and ammonium.   Two samples of disodium phosphate, one sample of dipotassium phosphate, and four samples of diammonium phosphate were found to be free from magnesium and sulphates.

Because of the expense of asparagine only 10 grams were used for the analysis. Six samples out of eleven were found to be free from magnesium, sulphates, and phosphates.   On account of the small amount of asparagine used for the analysis, it was decided to use a biological method of analysis for impurities.   Media were prepared using each sample of asparagine with different combinations of the inorganic constituents.   The pH of all of the media used in this research was between 6.8 and 7.2.   The following were prepared with 0.2 per cent asparagine:

1. Asparagine and 0.01 per cent phosphate.
2. Asparagine, 0.01 per cent phosphate, and 0.01 per cent Na_2SO_4.
3. Asparagine, 0.01 per cent phosphate, and 0.01 per cent $MgCl_2$.
4. Asparagine and 0.01 per cent $MgSO_4$.
5. Asparagine, 0.01 per cent phosphate, and 0.01 per cent $MgSO_4$.

The research to this point seems to indicate that the inorganic constituents necessary for fluorescence are magnesium, sulphate, and phosphates.   Assuming this to be true, then fluorescence in the above media would indicate certain impurities.   Positive results in number 1 would show the presence of sulphates and magnesium; in number 2, magnesium; in number 3, sulphates; in number 4, phosphates.   Number 5, of course, was used as a control.   The results for one of the five organisms used are listed in table 3.   The figures in parentheses indicate the number of hours necessary for the appearance of the maximum fluorescence

The results by the biological method did not show perfect agreement with the chemical method.   Sulphates were shown by the

biological method, in more samples, than by the chemical test. The reverse was true with magnesium. There were six of the eleven samples free from impurities when tested chemically. Five of these six were also free from impurities when tested biologically.

The purest samples of asparagine and dipotassium phosphate were selected as the samples to be used in all future media. Experiments had shown that there was little difference in the fluorescence produced with the sodium and potassium phosphates. The ammonium phosphates were not quite as satisfactory.

TABLE 3

Fluorescence with media made from different samples of asparagine (0.2 per cent), organism C

SAMPLE ASPARAGINE NUMBER	0 01 PER CENT K₂HPO₄	0 01 PER CENT K₂HPO₄ AND 0 01 PER CENT Na₂SO₄	0 01 PER CENT K₂HPO₄ AND 0 01 PER CENT MgCl₂	0.01 PER CENT MgSO₄	0 01 PER CENT K₂HPO₄, 0 01 PER CENT H₂SO₄ AND 0 01 PER CENT MgCl₂
1	−	−	−	−	++++(36)
2	−	++(84)	−	−	++++(36)
3	−	++(84)	−	−	++++(48)
4	−	+++(60)	−	−	++++(36)
5	+(60)	++(60)	+(72)	−	++++(36)
6	−	−	−	−	++++(36)
7	+(72)	+(72)	++(48)	−	+++(48)
8	−	++(72)	−	−	++++(36)
9	−	−	−	−	++++(36)
10	−	−	−	−	++++(36)
11	−	−	−	−	++++(36)

All of the sulphates, previously analyzed, were used for the preparation of media. Even if the sample did show considerable magnesium, it was used to show the effects of impurities. The media contained 0.2 per cent asparagine, 0.01 per cent dipotassium phosphate, and the sulphate in varying amounts. Similar media were made as above except that magnesium chloride was added; 0.0001, 0.001, and 0.01 per cent were used. With the media made from the purest sulphates, little fluorescence was found. Table 4 gives the results with the purest sulphate used, and table 5 gives the results with one of the C.P. analyzed sulphates which showed

the largest amount of magnesium present as impurity. Of course, all the media to which magnesium chloride was added gave excellent fluorescence.

In studying the media made from the different sulphates, it was found that the greater fluorescence was produced by the sample containing the most magnesium as impurity. It is possible that a sample of sulphate may contain enough magnesium to produce fluorescence and still show no magnesium on analysis, owing to

TABLE 4

Fluorescence in media containing 0.2 per cent asparagine, 0.01 per cent K_2HPO_4, and $(NH_4)_2SO_4$ no. 1

$(NH_4)_2SO_4$	ORGANISM A	ORGANISM C	ORGANISM E	ORGANISM F	ORGANISM G	ORGANISM H
per cent						
0.00001	—	—	—	—	—	—
0.0001	—	—	—	—	—	—
0.001	—	—	—	—	—	—
0.01	—	—	—	—	+(60)	—
0.1	+(48)	—	—	+(72)	−(60)	+(60)

TABLE 5

Fluorescence in media containing 0.2 per cent asparagine, 0.01 per cent K_2HPO_4, and K_2SO_4 no. 3

K_2SO_4	ORGANISM A	ORGANISM C	ORGANISM E	ORGANISM F	ORGANISM G	ORGANISM H
per cent						
0 00001		—	—	—	—	—
0.0001	+(60)	—	—	+(60)		+(72)
0.001	+(60)	+(72)	+(60)	+(60)	+(48)	+(72)
0.01	++(60)	+(60)	+(72)	+(72)	+(72)	+(48)
0 1	++(48)	++(72)	+(84)	+(84)	+(48)	+(48)

the solubility of the magnesium ammonium phosphate during the analysis.

As the result of over eleven hundred tests with the sulphates, it may be seen that magnesium is essential for the production of pigment. The media, containing the sulphates of the alkalies, gave little or no fluorescence, but in every case where magnesium chloride was added a good fluorescence was produced.

In order to prove definitely that the alkali sulphates will not suffice to produce fluorescence, it was decided to prepare a sulphate absolutely free from magnesium. This was done by selecting the purest ammonium hydroxide and sulphuric acid, and distilling each separately from an all quartz apparatus. The products were received in platinum dishes. The two were mixed in the proper proportions to form ammonium sulphate. A medium was made, using this pure sulphate with a pure sample of dipotassium phosphate and asparagine. The medium was first put in soft glass tubes, but a slight fluorescence, in some cases, was produced. Later quartz tubes and then pyrex tubes were used. Part of the results are recorded in table 6.

TABLE 6

Fluorescence in media made from pure $(NH_4)_2SO_4$ in different kinds of tubes (0.2 per cent asparagine, 0.01 per cent K_2HPO_4)

$(NH_4)_2SO_4$	SOFT GLASS No 1	SOFT GLASS No. 2	QUARTZ GLASS	PYREX GLASS	MEDIA PLUS 0.01 PER CENT MgCl₂
per cent					
0 00001	−	−	−	−	++(60)
0.0001	−	+(84)	−	−	+++(60)
0.001	+(72)	−	−	−	++++(48)
0 01	−	−	−	−	++++(48)
0.1	+(60)	+(72)	−	−	+++(48)

The results recorded in table 6 show that a medium made with ammonium sulphate in place of magnesium sulphate will not produce fluorescence. A slight fluorescence was produced when soft glass test tubes were used. It may be that a small amount of magnesium was dissolved from the glass.

The previous experiments have shown that magnesium must be present in a medium in order to produce fluorescence. The next question to be taken up was whether the sulphate was necessary. A number of C.P. analyzed samples of magnesium chloride and magnesium ammonium chloride were selected. These were analyzed for sulphates. Three out of seven samples of magnesium chloride gave no sulphates, and one sample out of three lots of magnesium ammonium chloride was free from sulphate.

.The above samples, together with some of the impure samples, were used in making up the test media. These media contained 0.2 per cent asparagine, varying amounts of potassium phosphate from 0.001 to 0.1 per cent, and ten different amounts (from 0.00001 to 0.5 per cent) of the magnesium chloride or magnesium ammonium chloride. This made fifty different media for each chloride used. The media, containing the chlorides free from sulphates, gave very little fluorescence, and such as did appear was probably due to impurities which could not be detected on analysis. In the media made with one sample of magnesium chloride pigment production was given in only three tubes out of the fifty tested. The samples of chlorides which contained the sulphate as impurity gave much more fluorescence especially when the larger amounts of magnesium chloride were used. To the fifty different media made from each chloride, 0.01 per cent sodium sulphate was later added. Each of these solutions was tested with the same organisms as above. In each medium, where the magnesium was present in amounts greater than 0.001 per cent, a good fluorescence was produced. This set of experiments shows clearly that the sulphate is necessary for pigment production.

Up to this point magnesium and sulphate have been shown to be indispensible for the production of pigment. The only other ingredient of the medium under question is the phosphate. Several sets of media were made with asparagine and magnesium sulphate, but no fluorescence was observed. When a phosphate was added, a good fluoresence was produced. Preliminary experiments had already shown that fluoresence was produced when either sodium, potassium, lithium, or ammonium phosphate was used in media with asparagine and magnesium sulphate. The intensity was not quite so great with the ammonium salt. A medium was then prepared with magnesium phosphate, asparagine, and magnesium sulphate. A fair fluorescence was produced with this combination. No attempt was made to analyze the chemicals for alkali metals.

It may be concluded, therefore, that in addition to an organic constituent such as asparagine, the medium must contain a phosphate, magnesium, and sulphate. This conclusion is not in

accord with the reports of the two most recent workers in this field.   Both Jordan and Sullivan found that magnesium was not necessary for the production of fluorescence.   It is practically impossible to obtain alkali sulphates free from magnesium. Also, there may be enough magnesium present to produce fluorescence and still not be detected on an analysis of a small amount of the sample.   In addition, it is apparent that a little magnesium may be dissolved from the test tubes.

In order to determine the amounts of the different constituents which, when present in a medium, will produce the best fluores-

TABLE 7

Organism A in media with 0.2 per cent asparagine; MgSO₄ and K₂HPO₄

MgSO₄	K₂HPO₄						
	0.0001 per cent	0 001 per cent	0 005 per cent	0 01 per cent	0 05 per cent	0 1 per cent	0 5 per cent
per cent							
0 00001	—	—	—	—	—	—	—
0.00005	—	—	—	—	—	+(84)	—
0 0001	—	—	+(72)	+(72)	+(84)	++(60)	—
0 0005	—	++(72)	+(72)	+(60)	+(72)	+(72)	—
0 001	—	+(72)	++(60)	+++(60)	+++(60)	++(72)	+(72)
0.005	—	++(60)	++(60)	++(48)	+++(60)	++(60)	+(72)
0.01	—	++(60)	+++(60)	++++(48)	++++(48)	+++(60)	++(60)
0.025	—	++(60)	+++(48)	+++(48)	+++(48)	++++(48)	++(60)
0 05	—	++(60)	+++(48)	++++(48)	++++(36)	++++(48)	+(72)
0.1	—	++(60)	+++(60)	++++(48)	+++(48)	+++(60)	+(72)
0.2	—	++(60)	+++(60)	+++(48)	+++(48)	++(60)	+(72)
0.5	—	++(60)	++(60)	++(48)	+++(60)	+(60)	+(72)

cence, a series of experiments was run.   Media were, therefore, made with varying amounts and combinations of the three ingredients.   Table 7 shows the results with 0.2 per cent asparagine with one of the organisms   Media containing 0.1, 0.3, 0.5, and 0.7 per cent asparagine were also used.

The fluorescence in the lower percentages of asparagine was about equally good.   The medium containing 0.3 per cent was probably a little better.   The pigment production was very good when the phosphate content was between 0.005 and 0.1 per cent,

and when the magnesium sulphate was between 0.001 and 0.2 per cent. The best and quickest pigment production was in the media containing 0.3 per cent asparagine, 0.05 per cent dipotassium phosphate, and 0.05 per cent magnesium sulphate. Considering all of the tests similar to the one given in table 7, very little or no fluorescence was produced when the phosphate content was less than 0.0005 per cent or when the magnesium was less than 0.00005 per cent. There was, of course, some variance with different organisms.

In light of the above the following medium is given as the most satisfactory for the production of fluorescence:

Magnesium sulphate, anhydrous............................	0.5 gram
Dipotassium phosphate, anhydrous......................	0.5 gram
Asparagine...	3.0 grams
Distilled water.. 1000	cc.

CONCLUSION

1. A comprehensive study of the inorganic constituents necessary for the production of bacterial fluorescence has been reported. Over 4500 inoculations have been made.

2. The presence of magnesium, phosphate, and sulphate has been found to be essential for pigment production.

3. Highly purified chemicals may contain enough impurities to cause the production of fluorescence.

4. Media in some kinds of ordinary soft glass test tubes may, apparently, dissolve enough magnesium to permit of the production of some fluorescence. The use of pyrex or quartz test tubes eliminated this source of magnesium as shown by the absence of pigment formation in magnesium-free media when used in such tubes.

5. The most satisfactory medium for the production of bacterial fluorescence has been suggested.

6. The production of fluorescence may be used in place of chemical tests as a very delicate method for the detection of sulphates, phosphates, and magnesium.

REFERENCES

BERGEY, D. H. 1930 Manual of Determinative Bacteriology, 3rd edition, Williams and Wilkins, Baltimore.

GESSARD, C. 1892 Sur la fonction fluorescigène des microbes. Ann. de l'Inst. Pasteur, 6, 801.

HUEPPE, F. 1880 Etudes sur le lait blue. Cohn's Beiträge zur Biologie de Pflanzen, 3, 1880. Original not seen.

JIROU, J. 1901 Sur les Bacilles fluorescents et le pyocyanique. Jour. de Physiol. et le Pathol. Gén., 3, 188.

JORDAN, E. O. 1899 Production of fluorescent pigment. Botanical Gaz., 27, 19.

LEPIERRE, C. 1895 Recherches sur la fonction fluorescigène d'un Bacille fluorescent pathogène. Ann. de l'Inst. Pasteur, 9, 643.

NÄGELI. Original not seen. See THUMM. Arbeiten aus dem Bakt. Inst. der Technischen Hochschule zu Karlsruhe, 1, 1895.

SULLIVAN, M. 1905 Synthetic culture media and the biochemistry of bacterial pigments. Jour. Med. Res., 14, 109.

TANNER, F. W. 1918 A study of green fluorescent bacteria from water. Jour. Bact., 3, 63.

THUMM, K. 1895 Beträge zur Biologie der fluorescirenden Bakterien. Arbeiten aus dem Bakt. Inst. der Technischen Hochschule zu Karlsruhe, 1, 291.

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OBSERVATIONS ON VARIANTS DERIVED FROM TWO STRAINS OF STREPTOCOCCUS HEMOLYTICUS[1]

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Within the last decade workers have become increasingly interested in organisms which, in the course of their growth, show variations from their usual characteristics. Recent studies indicate that the *Streptococcus hemolyticus* and its colony are subject to change.

Valentine and Krumwiede (1922) observed the sudden and spontaneous appearance of green colonies of one of twenty-one strains of *Streptococcus hemolyticus*. The green colonies bred true to type in subculture and were serologically identical with the original hemolytic strain. Walker (1923) described two types of colonies of *Streptococcus hemolyticus* isolated from the pleural fluid of a rabbit which had been previously inoculated. One type, which he termed *normal*, grew as a sediment in broth, was moderately hemolytic, and killed mice in a dilution of 10^{-4}. The other, or *moist* type, which after fourteen subcultures on blood agar became identical with the *normal*, gave diffuse growth in broth, was more markedly hemolytic, and had a virulence of 10^{-5}. Griffith (1927) noted rough, soft and mucoid-opaque colonies following the twenty-four-hour incubation of *Streptococcus hemolyticus* on a nutrient agar made with trypsinized meat broth and oxalated horse blood. Andrewes (1928) described *R* and *S* colonies isolated from single strains. The *R* colony was subsequently found to be virulent, the *S* colony avirulent. His agglutination tests suggested that the smooth forms of different

strains were serologically identical, while the rough forms showed serological variation.

Few such cases of spontaneous change have been reported and for the most part variations have been brought about only after persistent subculture and discriminative selection of colonies. Howell and Beverley (1926) reported that a strain of *Streptococcus hemolyticus* isolated with a *Streptococcus viridans* from the blood of a patient with an acute malignant endocarditis, lost its hemolyzing quality after seven months alternate culture on blood-agar and ascitic-glucose-agar, and that it remained constantly anhemolytic thereafter. Cowan (1922) cultured *Streptococcus hemolyticus* alternately in serum broth and on agar and selected for subculture the colonies showing the greatest degrees of roughness or smoothness. After several months she found that she had obtained two distinct variants: a rough, avirulent type; and a smooth, virulent variety. Todd (1928) found that *Streptococcus hemolyticus* became attenuated when cultivated on agar slants. After prolonged cultivation he was able to obtain a *glossy* form which was permanently attentuated, and a rough or *matt* form which could be exalted by growth in serum or attenuated by further cultivation on agar. He also noted that, while most freshly isolated cultures contain only the matt form, the glossy forms subsequently take their place because of their greater ability to multiply rapidly. Further studies carried on by Todd and Lancefield (1928) caused them to classify the *Streptococcus hemolyticus* into three groups: two varieties of matt colonies equally rich in type-specific substance but differing in virulence for mice, and one type of glossy colony characterized by almost complete absence of the type-specific substance. This latter type, whose form was highly stable, was produced by prolonged cultivation either on agar slants, or in broth, or in undiluted homologous anti-type-specific serum of high titer. The toxic filtrates of all three forms were approximately equal in strength when tested in skin-reactive individuals. Eagles (1928), working with two stock strains of *Streptococcus hemolyticus*, was able to obtain, by selective subculture of colonies from serum agar to serum broth, colonies which were completely smooth and colonies which were very rough.

In determining the virulence of these strains he found that the rough variety of one strain, and the smooth form of the other, were virulent. Tunnicliff (1930) after studying colonies of *Streptococcus hemolyticus* on chocolate agar (pH 7.0) reported that typical cultures from erysipelas, when incubated at 34°C., produced a vivid green color after from twenty-four to forty-eight hours incubation; cultures from scarlet fever did not produce a similar change. She noted also that colonies of *Streptococcus hemolyticus* from scarlet fever were slightly granular and conical; that those from erysipelas were smooth and convex, later becoming flat and rough; and that those from septic sore throats were very rough, indented and conical or convex. Grinnell (1928) working with single cell strains, concluded that certain cultures of *Streptococcus hemolyticus* isolated from scarlet fever might give rise to colonies of the alpha type if grown either in broth containing 20 per cent normal horse serum, or on blood agar slants incubated at 40°C. These green colonies were smooth and grew with uniform turbidity in broth and remained true to type.

SOURCE OF MATERIAL

Eighteen strains of *Streptococcus hemolyticus* were studied immediately after their isolation with especial reference to colony morphology. Of 11 isolated from throat cultures, 7 were from cases of scarlet fever, 3 from streptococcal sore throats, and one from an acute follicular tonsillitis. Five were isolated from blood cultures; one from pleural fluid; and one from an area affected with diabetic gangrene.

EXPERIMENTAL PROCEDURE

The method used for the study of the colony morphology of the *Streptococcus hemolyticus* was similar to that described by Todd in 1928. The organisms were isolated in pure culture on blood-agar plates and were incubated for twenty-four hours at 37°C. Several colonies were then seeded on a chocolate-agar plate. These plates were made by adding 10 cc. of normal rabbit's blood to 250 cc. of melted infusion agar, which had been cooled to 45°C. The flask was then immersed in a water bath at 80°C. for from five

to seven minutes, cooled, and the contents poured into sterile petri dishes. After incubation for twenty-four hours at 37°C., the colonies were studied grossly and under the low power of the microscope by reflected light. Throughout this study the terms employed by Todd (1928) have been used to describe colony morphology. A *matt* colony is one whose growth on blood agar is characterized by a rough surface and the ability to remain intact when pushed by a loop, and whose growth in broth is characterized by granules. A *glossy* colony, on the other hand, presents a smooth surface on solid media, disintegrates when touched, and grows diffusely in broth.

Of the eighteen different strains of *Streptococcus hemolyticus* studied on chocolate-agar all but one were of the matt variety. The only colony which differed from these was isolated from pleural fluid, and on cultivation appeared to be moist and smooth, similar to Griffith's (1927) third type. After four successive transplants on infusion and chocolate agar the surface showed roughness and the colony became identical in appearance with the matt type.

A study was then undertaken to see if all these matt varieties of the *Streptococcus hemolyticus* could be induced to give rise to glossy forms after prolonged cultivation on infusion agar. The organisms were grown at 37°C. for twenty-four hours on plates of infusion-agar, and were subcultured without discrimination as to colony morphology to a second agar plate and to a chocolate-agar plate. After twenty-four hours' incubation, the colonies from the plain agar were again subcultured to plain and to chocolate-agar, while those on the first chocolate-agar plate were studied grossly and under the low power of the microscope by reflected light. As a control, the matt variety was carried on blood-agar, and transplants were frequently made to chocolate-agar to see if any change had taken place.

The method described by Dick and Dick (1925) was used to determine the amount of toxin produced. Several colonies from a blood-agar culture of the strain to be tested were transplanted to tubes containing 1 per cent normal rabbit-blood-broth, and were incubated for four days at 37°C. The cultures were then run

through a Chamberland filter, tested for sterility, and 0.1 cc. amounts of dilutions made in sterile physiological salt solution were injected intradermally in both Dick-positive and Dick-negative individuals.

When a change in colony morphology appeared, the variant was studied serologically and by further cultural methods. Antiserum was prepared by the intravenous inoculation of rabbits with heat-killed organisms of both the variant and the original strain. The twenty-four-hour growth of a chocolate agar slant washed with 2 cc. of saline was used for this procedure, after having been subjected to a temperature of 60°C. for one hour and tested for sterility. Rabbits were inoculated on four successive days of each week, and rested for three days, the doses used ranging from an initial amount of 0.1 cc. to the final amount of 1.5 cc. The serum was tested for agglutinins at intervals of two weeks. To insure a homogeneous antigen for these agglutination tests, the smooth variants were grown for twenty-four hours in 35 cc. amounts of filtered chocolate-broth. This broth was made by adding 10 cc. of defibrinated normal rabbit's blood to 50 cc. of infusion broth and heating at 80°C. for ten minutes. This was then filtered through a sterile Chamberland filter and an equal part of infusion-broth was added to the filtrate. The growth of the variants was centrifuged and most of the supernatant fluid was decanted. The organisms were resuspended in enough of the supernatant fluid to give the required density. Great difficulty was experienced in obtaining a homogeneous suspension of the matt variety of the *Streptococcus hemolyticus*, and after several experiments the method described by Tunnicliff (1920) was adopted with certain modifications. The organisms were grown in a liquid medium of pH 7.6 containing one part ascitic fluid and four parts 0.2 per cent glucose broth. The cultures were incubated twenty-four hours, centrifuged and enough of the supernatant fluid retained to make an antigen suspension of the required density. The rabbit serum to be tested was diluted with infusion broth 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, and 1:5120. The agglutination tests were placed in a 56°C. water bath for two hours and read after standing at ice-box temperature for eighteen hours.

Seven-hour cultures in filtered chocolate-broth of both the matt and glossy variants of the *Streptococcus hemolyticus* were used in determining the virulence. Mice were inoculated intraperitoneally with 1.0 cc. of dilutions of this culture ranging from 10^{-1} to 10^{-6}. After death, cultures were made of the peritoneal exudate and the heart's blood.

The further cultural methods employed in the study of the glossy variants obtained were carried on to determine if reversion to the original matt type would occur, or if further changes would take place. The organisms were grown on agar containing 4 per cent rabbit blood; in filtered chocolate-broth; in broth containing 1 per cent normal rabbit serum; and in broth containing 1 per cent serum from a rabbit having a titer of 1:5120 for the organism under study. In all cases transplants were made after each twenty-four hour incubation, and each culture was studied grossly and after its subculture on chocolate-agar. The original matt strain was also carried on blood-agar; in infusion-broth; in 1 per cent normal serum-broth; and in broth containing 1 per cent serum from a rabbit having a titer of 1:20 for the strain under observation and a titer of 1:5120 for its corresponding glossy variant. In this case also the cultures were observed grossly and following subcultivation on chocolate-agar.

RESULTS

Of the eighteen strains of *Streptococcus hemolyticus* carried on infusion-agar without blood for from 18 to 170 transplants, only two strains showed permanent changes in colony morphology. In addition, they had acquired the ability to change the color of chocolate-agar from a reddish-brown to a striking greenish yellow-brown. This change in color extended for a considerable distance from the immediate vicinity of the colony and in heavily seeded plates involved the whole medium. Several strains showed a temporary ability to change the medium to a dark-green color in a narrow zone about individual colonies, but this was not related to change in colony morphology. In no case were changes noted in the colonies grown on agar to which whole unheated rabbit's blood had been added.

Strain A

After seventy-two transplants on infusion agar without blood, strain A, which, when originally isolated from the throat of a patient suffering from scarlet fever, had been composed only of matt colonies (figs. 1 and 2), showed changes in colony morphology when studied on chocolate-agar. The colonies no longer had rough surfaces but were smooth, slightly moist and glistening (figs. 3 and 4), and at the same time changed the color of chocolate-agar to a greenish yellow-brown. The organisms of which
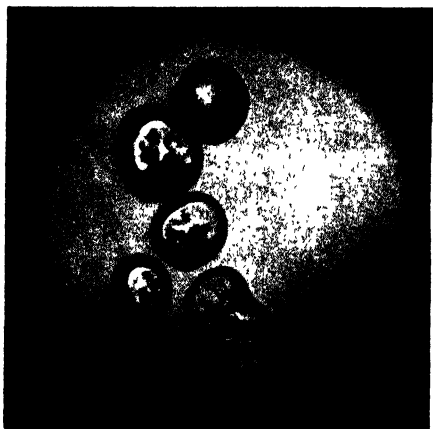
FIG. 1   FIG. 2

FIG 1. STRAIN A. MATT COLONIES. TWENTY-FOUR-HOUR GROWTH
FIG. 2. STRAIN A. MATT COLONIES. FORTY-EIGHT-HOUR GROWTH
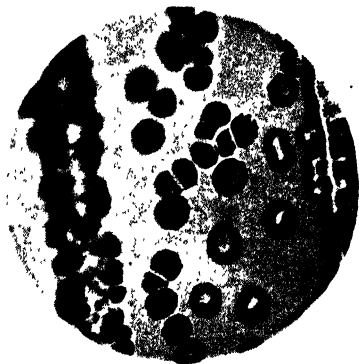
the colonies were composed were highly pleomorphic and Gram-variable. They were bile-insoluble and were not agglutinated by antipneumococcus sera, types I to XXVI. Like the original ones from which they were derived, they were avirulent when injected into the peritoneal cavities of white mice. A marked change was noted in the ability to produce a soluble toxin. The original strain produced a toxin of sufficient potency to give a markedly positive skin test in the arm of a Dick positive individual in a dilution of 1:1000, but a dilution of 1:50 of the filtrate of the glossy variant was required to give a similar reaction in the

same individual. These glossy colonies failed at first to grow on infusion-agar to which normal unheated rabbit's blood had been added, and it was only after many attempts and after they had been carried on plain infusion agar for 149 transplants, that small greenish glistening colonies appeared. These colonies subcultured on blood-agar remained constant in character until the ninth transplant when a clear zone of hemolysis appeared outside the green coloration. The next change appeared at the thirteenth transplant when the colony lost its green halo. On the fifteenth transplant the colony reverted to the type found on the ninth and

FIG. 3   FIG. 4

FIG. 3. STRAIN A. GLOSSY COLONIES. TWENTY-FOUR-HOUR GROWTH
FIG. 4. STRAIN A. GLOSSY COLONIES. FORTY-EIGHT-HOUR GROWTH

retained these characteristics until the forty-fourth. At that time the hemolytic zone was lost and the individual colonies, although smooth, resembled the *Streptococcus viridans*, a character which was retained throughout the remainder of the study. Throughout these variations the ability to change the color of chocolate-agar from a reddish-brown to a greenish yellow-brown was constantly present. Immune serum prepared by the intravenous inoculation of rabbits with the heat-killed glossy variant of this strain agglutinated its homologous antigen in a dilution of 1:5120 with a large flaking type of agglutination, and aggluti-

nated the corresponding matt variety in a dilution of 1:20 with fine flakes.

In order to ascertain whether, under certain circumstances, the glossy variant derived from strain A could be induced to revert to the original matt type or give rise to further variants, it was grown on three other types of media: 20 successive subcultures were made in 5 cc. amounts of filtered chocolate-broth; 20 in infusion-broth containing 1 per cent serum from a rabbit with an aggluti-nation titer of 1:5120 for this organism; and 7 transplants in infusion-broth containing 1 per cent normal rabbit serum.   In all
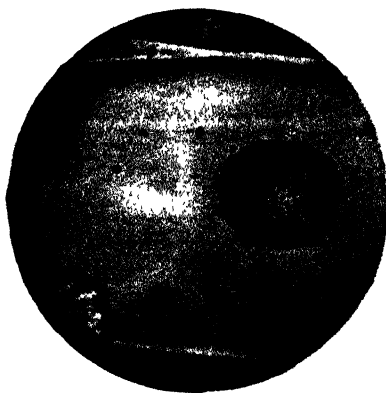
FIG. 5. STRAIN A. PEBBLY COLONY. TWENTY-FOUR-HOUR GROWTH

cases the cultures were studied grossly and were subcultured on chocolate-agar plates for examination under the low power of the microscope.   No change was demonstrated in the cultures made in filtered chocolate-broth or in the broth containing anti-serum, but after five transplants in the normal serum broth a very rough colony appeared together with the glossy type.   This colony (fig. 5) was slightly larger in size, appeared granular even to the naked eye and under the microscope the pebbly surface showed furrows and irregularities.   Only occasionally did it present the peaked surface of the matt type.   On subculture it was shown that this new type of colony had the ability, as did the glossy variant from

which it was derived, to change the color of chocolate-agar to a greenish yellow-brown. The growth of this form in chocolate-broth and in normal-rabbit-serum broth was granular. When transplanted to blood-agar it yielded a pure culture of pebbly rough colonies, but when subcultured on chocolate-agar a mixed culture of glossy and rough colonies was almost invariably obtained. Subcultures on blood- and chocolate-agar made by a discriminative selection of the colonies appearing to have the greatest degree of roughness yielded after ten transplants in each case a rough or *pebbly* form which seemed to be constant, although the growth on blood agar always tended to present a slightly rougher picture than on chocolate-agar. The pebbly colony was found to be serologically identical with the glossy variant from which it was derived.

Strain B

The change exhibited by strain B, originally isolated from the blood culture of a diabetic patient with septicemia, was similar. Smooth glossy colonies were first noted after 49 transplants on plain agar, and at the same time the color of chocolate-agar was changed to a greenish yellow-brown. The original virulence of this streptococcus had been such that 1.0 cc. of a 1 : 100 dilution of a seven-hour broth culture killed a mouse sixty hours after intra-peritoneal injection, but the organisms of the glossy variant were completely avirulent. Toxin production of the glossy type was almost negligible, a dilution of 1 : 10 being required to give a posi-tive skin test in a Dick-positive individual although the toxin pro-duced by the matt type had been sufficient to give a markedly positive reaction in the same individual in a dilution of 1 : 100. Subcultures on infusion-agar with blood failed to grow until the colonies had been carried on the plain infusion-agar for 75 trans-plants. At that time a small green colony with a zone of hemo-lysis about it appeared on subculture. After 36 subcultures on blood-agar the clear zone was replaced by green coloration and the colonies appeared similar to the *Streptococcus viridans*, although preserving their glossy form and their ability to change the color of chocolate agar. No attempt was made to cause re-

version or to bring about further changes in this strain as was done in the case of strain A.

At this point it was thought that a study of throat cultures of patients convalescing from *Streptococcus hemolyticus* infections might show the presence of either glossy or pebbly forms, in addition to the normal matt form of colony.   Therefore, throat cultures were made on blood agar plates, incubated for twenty-four hours at 37°C.   An attempt was made to isolate to a second blood agar plate all colonies found resembling any of the forms of *Streptococcus hemolyticus*.   These colonies were transplanted to chocolate-agar where they were studied both grossly and microscopically.   Although daily throat cultures from 5 cases of scarlet fever and from 3 cases of erysipelas were studied over a period of from two to six weeks, no colonies of either the glossy or the pebbly type were found, and in no instance did the colony of Streptococcus under observation demonstrate the ability to change the color of chocolate agar.

SUMMARY

Of the eighteen matt strains of *Streptococcus hemolyticus* carried on infusion-agar without blood for from 18 to 170 transplants, only two strains exhibited ability to give rise to glossy variants. One of these strains required 49, the other 75, transplants before showing any glossy colonies.   These glossy derivatives, growing with difficulty in the presence of whole, unheated blood and unable to produce as potent a toxin as their matt forms, acquired the ability to change the color of chocolate-agar from a dark reddish-brown to a light greenish yellow-brown, a phenomenon not previously described.

One of the glossy variants, when grown in 1 per cent normal rabbit serum broth for seven transplants, gave rise to a very rough or *pebbly* form which was serologically identical with the glossy form and preserved its ability to change the color of the chocolate-agar.

In the study of daily throat cultures taken over a period of from

two to six weeks from five patients with scarlet fever and three with erysipelas, all colonies of *Streptococcus hemolyticus* were of the matt type and no colonies of the glossy or pebbly forms were isolated.

REFERENCES

ANDREWES, F. W. 1928. Specific and group agglutination against the hemolytic streptococci. Jour. Path. and Bact., **31**, 132.

COWAN, M. L. 1922. Variation phenomena in streptococci, with special reference to colony form, hemolysin production, and virulence. Brit. Jour. Exper. Path., **3**, 187.

DICK, G. F., AND DICK, G. H. 1925. Method of recognizing scarlet fever Streptococcus by means of specific toxin production. Jour. Amer. Med. Assoc., **84**, 802.

EAGLES, G. H. 1928. Colony variants in haemolytic streptococci. Brit. Jour. Exper. Path., **9**, 330.

GRIFFITH, F. 1927. Haemolytic streptococci in relation to scarlet fever. Jour. Hyg., **26**, 363.

GRINNELL, F. B. 1928. Streptococcus studies: Streptococcus viridans derived from single cell strains of *Streptococcus hemolyticus*. Jour. Bact., **14**, 117.

HOWELL, K., AND BEVERLEY, D. A. 1926. Apparent mutation of streptococci from acute malignant endocarditis. Jour. Infect. Dis., **39**, 12.

TODD, E. W. 1928. Further observations on the virulence of haemolytic streptococci, with special reference to morphology of the colonies. Brit. Jour. Exper. Path., **9**, 1.

TODD, E. W., AND LANCEFIELD, R. C. 1928. Variants of hemolytic streptococci; their relation to type-specific substance, virulence and toxin. Jour. Exper. Med., **48**, 751.

TUNNICLIFF, R. C. 1930. Streptococci from scarlet fever, erysipelas and septic sore throat. Jour. Amer. Med. Assoc., **94**, 1213.

VALENTINE, E., AND KRUMWIEDE, C. 1922. The loss of hemolytic capacity by a fraction of a culture of a hemolytic streptococcus without change in agglutination characteristics. Jour. Exper. Med., **36**, 157.

WALKER, J. E. 1923. Variations in *Streptococcus hemolyticus* on animal passage. Jour. Infect. Dis., **32**, 287.

THE SEROLOGIC AGGLUTINATION OF BACILLUS SORDELLII AND CLOSTRIDIUM OEDEMATOIDES

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The work of Humphreys and Meleney (1928) and of Hall, Rymer and Jungherr (1929) firmly established the identity of *Bacillus Sordellii* and *Clostridium oedematoides* on the basis of cultural and morphologic comparisons and toxin-antitoxin cross protection tests. Hall (1929) later identified two cultures of unknown anaerobic bacilli sent to him by Dr. L. R. Vawter of Reno, Nevada as *B. Sordellii* in the same manner.

In none of the preceding comparative studies, however, was the possibility of serologic identification by means of the agglutination reaction satisfactorily demonstrated. Sordelli (1923) evidently tried agglutination, for he said, "l'agglutination n'a pas permis d'etablir ni difference, ni analogie," but he gave no experimental results. Humphreys and Meleney (1928) stated that, "Agglutination reactions in this group" (the pathogenic clostridia) "are notoriously unreliable, owing, on the one hand, to the indefinite number of subgroups likely to be encountered, and on the other, to their frequent tendency to spontaneous agglutination." The work reported in this paper is a continuation of the comparative studies reported by Hall, Rymer and Jungherr in 1929, and deals with cross agglutination studies on the eight available strains of *B. Sordellii* and *C. oedematoides*.

The following cultures were investigated:

B. Sordellii 1302, separated from Sordelli's "*B. oedematis sporogenes* F9" as "solid" dissociate by J. P. Scott. Renamed by Hall and Scott (1927); pathogenic.

B. Sordellii 1303, separated from Sordelli's *"B. oedematis-sporogenes* F9" as "fluffy" dissociate by J. P. Scott.   Renamed by Hall and Scott; pathogenic.

Clostridium oedematoides 1316, received from Frank Meleney, Presbyterian Hospital, New York, as "strain no. 1 from patient;" pathogenic.

Clostridium oedematoides 1317, received from Frank Meleney, Presbyterian Hospital, New York, as "strain no. 2 from catgut;" pathogenic.

Clostridium oedematoides 1318, received from Frank Meleney, Presbyterian Hospital, New York as "strain no. 3 from catgut;" pathogenic.

B. Sordellii 1322, isolated from Sordelli's *"B. oedematis-sporogenes* 82." Renamed by Hall and Scott; non-pathogenic.

B. Sordellii 2782, received from L. R. Vawter, University of Nevada, Reno, Nevada, as culture 6618 from bovine mesenteric lymph gland; pathogenic.

B. Sordellii 2783, received from L. R. Vawter, University of Nevada, Reno, Nevada, as culture 7441 from bovine liver infarct; pathogenic.

IMMUNIZATION OF ANIMALS

Our first problem was the production of potent agglutinating sera in rabbits.   Due to the toxicity of the cultures, considerable difficulty was encountered.   Jungherr (1927) and Hall, Rymer and Jungherr (1929) reported the same trouble, especially with intravenous injections.

Two attempts were made to immunize rabbits with small doses of whole culture without first producing antitoxic immunity; these were unsuccessful resulting in the early death of the animals, even when amounts as small as 0.01 cc. were used subcutaneously.

Attempts to use killed cultures of bacilli were unsuccessful. Cultures killed by heat have been used by Tulloch and Bauer and Meyer (1926) in the preparation of anti-tetanus agglutinating sera, and by Schoenholz and Meyer (1923) to produce anti-botulinus sera.   Jungherr (1927) claims to have produced strong agglutinins for *B. Sordellii* and *C. oedematoides* by this method, although he could not demonstrate any reaction after two injections, as had Bauer and Meyer with tetanus; in his hands at least 15 injections totaling 19 cc. were necessary.   We were unable to demonstrate agglutinins after a total of 45 cc. of antigen had been given.

TABLE 1

Immunization of rabbit R19 against C. oedematoides 1318

DATE	WEIGHT	DOSE	MATERIAL	ROUTE
1929	*grams*	*cc.*		
August 10......	3,250	1 (1:50)	Toxin	Subcutaneous
August 17......................	3,250	1 (1:50)	Toxin	Subcutaneous
August 24.	3,350	1 (1:40)	Toxin	Subcutaneous
August 31......................	3,425	1 (1:30)	Toxin	Subcutaneous
September 7........	3,450	1 (1:25)	Toxin	Subcutaneous
September 11..................	3,500	1 (1:25)	Toxin	Subcutaneous
September 15.....	3,550	1 (1:25)	Toxin	Subcutaneous
September 20..................	3,550	1 (1:10)	Toxin	Subcutaneous
September 25.....	3,600	1 (1:25)	Toxin	Subcutaneous
October 10	3,625	1 (1:25)	Toxin	Subcutaneous
October 20	3,650	1 (1:25)	Toxin	Subcutaneous
November 1...	3,675	1 (1:25)	Toxin	Subcutaneous
November 15..........	3,600	1 (1:10)	Toxin	Subcutaneous
November 30..................	3,675	1 (1:5)	Toxin	Subcutaneous
December 15..................	3,675	1 (1:5)	Toxin	Subcutaneous
1930				
January 3....................	3,800	1 (1:5)	Toxin	Subcutaneous
January 18	3,700	0.3	Toxin	Subcutaneous
January 24....	3,700	0.5	Toxin	Subcutaneous
February 4....................	3,700	1	Toxin	Subcutaneous
February 8....................	3,750	0.4	Toxin	Subcutaneous
February 13...................	3,750	0.8	Toxin	Subcutaneous
February 17.......	3,750	1.2	Toxin	Subcutaneous
February 22..................	3,700	2	Toxin	Subcutaneous
March 1.....................	3,700	3	Toxin	Subcutaneous
March 11....................	3,710	5	Toxin	Subcutaneous
March 17.....................	3,725	10	Toxin	Subcutaneous
			WHOLE CULTURE AGE	
			hours	
March 25.....................	3,675	0.1	48	Subcutaneous
March 31.....................	3,750	0.2	48	Subcutaneous
April 5.......................	3,750	0.4	48	Subcutaneous
April 19......................	3,675	0.6	48	Subcutaneous
April 26......................	3,600	0.8	48	Subcutaneous
April 30......................	3,650	1	48	Subcutaneous
May 5........................	3,650	2	48	Subcutaneous
May 10.......................	3,650	5	48	Subcutaneous
May 15.............	3,750	5	72	Subcutaneous
May 20.......................	3,700	5 (×2)	72	Subcutaneous
May 24.......................	3,675	5 (×3)	48	Subcutaneous

TABLE 1—*Concluded*

DATE	WEIGHT	DOSE	WHOLE CULTURE AGE	ROUTE
1930	*grams*	*cc.*	*hours*	
May 29......................	3,750	5 (×3)	48	Subcutaneous
June 3.......................	3,700	5 (×4)	72	Subcutaneous
June 15.................	3,600	Bled about 15 cc. from ear; 1:2,000		
June 23......................	3,650	5 (×4)	48	Subcutaneous
June 27......................	3,650	5 (×4)	72	Subcutaneous
July 1	3,700	5 (×4)	24	Subcutaneous
July 9.......................	3,600	5 (×4)	72	Subcutaneous
July 16......................	3,600	5 (×4)	24	Subcutaneous
July 18......................	3,600	5 (×4)	48	Subcutaneous
July 25......................	Bled about 15 cc. from ear; 1:200			

Total material injected: Toxin, 25.38 cc.; whole culture, 195.1 cc.

Note. Numbers in parenthesis following dosage of toxin indicate dilution; those following the dosage of whole culture indicate concentration, e.g., 5 (×4) means the growth of 20 cc. of broth culture, centrifugalized and resuspended in 5 cc. of broth for injection.

TABLE 2

Summary of injections

RABBIT	TOXIN	WHOLE CULTURE	NUMBER OF INJECTIONS
	cc.	*cc.*	
261 anti 1302	29.36	33.4	32
208 anti 1303	19.76	70.5	30
274 anti 1316	25.91	192.0	41
232 anti 1317	26.06	190.5	49
R19 anti 1318	25.38	195.1	45
206 anti 1322		97.0	11

The use of formalinized antigen prepared by the method of Weinberg and Barotte (1929) also yielded negative results in our hands.

Antitoxic immunity was first produced by the injection of graded doses of filtered toxin; when the animal could tolerate 5 to 10 cc. of undiluted toxin subcutaneously, graded doses of whole culture were injected to produce agglutinin formation, but in the case of the non-pathogenic strain, 1322, it was unnecessary to start with the filtrate.

The protocol presented in table 1 is representative of the procedure used successfully with the virulent strains.

Table 2 shows the total amounts of toxin and of whole culture used in immunizing each animal and the number of injections.

TECHNIC OF AGGLUTINATION TESTS

Antigenic suspensions were prepared by filtering forty-eight-hour glucose broth cultures through loose cotton to remove clumps. Attempts were made to use centrifugalized cultures resuspended in normal saline containing 0.3 per cent tricresol, hoping that this method would avoid the formation of gas in the tubes, but such preparations failed to agglutinate in controlled tests.

For the preliminary titrations of the sera, three dilutions, namely, 1:10, 1:100, and 1:1000, were made. One cubic centimeter of each dilution was pipetted into a test tube, 100 by 13 mm., and 1 cc. of the homologous antigen added, making the final dilutions 1:20, 1:200 and 1:2000. A control tube containing 1 cc. normal saline solution and 1 cc. of antigen was also set up. Results were read after three hours in the incubator at 37°C.

In making the final tests, the same general scheme was followed as in the preliminary tests, but the dilutions of serum used were: 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560 and 1:5120; thus the final dilutions ranged from 1:20 to 1:10,240.

PRELIMINARY TITRATION OF SERA

The results of the preliminary titrations are shown in table 3. The differences in time required to produce strong agglutinins for the various strains were striking. Thus, a satisfactory titer was reached for the non-pathogenic South American strain 1322 in about six weeks. The pathogenic South American strains, 1302 and 1303, and the New York strains, 1316 and 1317, produced good sera in five to six months. The New York strain 1318 gave us unusual difficulty; rabbit after rabbit was started only to die before the immunization was complete. This finally made us extremely cautious in pushing the dosage so that the eleven

TABLE 3

Preliminary homologous agglutination titers in serums bled at different times

SERUM NUMBER	IMMUNIZATION BEGUN	DATES SERUM DRAWN						
		A July 6, 1928	B August 1, 1928	C September 10, 1928	D November 27, 1929	E April 21, 1929	F June 15, 1930	G July 25, 1930
261 anti 1302	March 7, 1928	++ 1:200	+++ 1:2,000	++ 1:2,000				
208 anti 1303	March 17, 1928	- 1:20	+ 1:20			± 1:160		
274 anti 1316	March 8, 1928	+ 1:200	± 1:2,000	+ 1:2,000	+++ 1:2,000	± 1:20		
232 anti 1317	March 8, 1928	+ 1:200	- 1:20	+ 1:2,000	+ 1:2,000	- 1:20		
206 anti 1322	October 5, 1928				+ 1:2,000	- 1:20		
R19 anti 1318	August 10, 1928				+++ 1:2,000	± 1:80	+ 1:2,000	++ 1:200

Note: All sera were titrated within four days after the bleeding.

The figures in the table indicate the highest dilution in which a positive agglutination appeared and the intensity of the reaction. Complete agglutination would be indicated by ++++. No preliminary tests were made at dilutions higher than 1:2,000.

TABLE 4

Cross agglutination tests with serum 274 anti C. oedematoides 1816

Serum drawn November 27, 1928.   Test made December 4, 1928.

STRAIN	DILUTION OF SERUM										
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	Control
B. Sordellii........ 1302	+++	+++	+++	++	-	-	-	-	-	-	-
B. Sordellii........ 1303	+++	+++	+++	+++	-	-	-	-	+	±	-
C. oedematoides.... 1316	+++	+++	+++	+++	+	-	+++	++	-	-	-
C. oedematoides.... 1317	+++	+++	+++	+++	++	++	++	++	+	++	-
C. oedematoides.... 1318	+++	+++	++	+++	++	++	++	++	++	++	-
B. Sordellii........ 1322	+++	+++	+++	+++	++	++	++	++	++	+	-
B. sporogenes...... 10	-	-	-	-	-	-	-	-	-	-	-
B. tyrosinogenes... 106	-	-	-	-	-	-	-	-	-	-	-
B. Novyi........... 140	-	-	-	-	-	-	-	-	-	-	-

months actually consumed were possibly even longer than necessary, but they caused a considerable delay in completing the tests.

CROSS AGGLUTINATION

Within a few days after the homologous titer of each serum was found to have reached 1:2000, the cross agglutination tests were made. The early protocols included only the three South American and the three New York strains of *B. Sordellii* described by Hall, Rymer, and Jungherr, and certain representative strains of heterologous species, *B. tyrosinogenes* 106 and *B. sporogenes* 10 which were included because of their cultural resemblance to *B. Sordellii*, and *B. Novyi* 140 which was included because of the pathologic resemblance of the lesions produced by it in animals. These strains have already been described as to origin by Hall (1922a, 1922b). The cross tests of all sera (except that prepared against 1318) upon all of these strains were completed before January 1, 1929. The Nevada strains, 2782 and 2783, were not received until February, 1929, and not identified until April; we decided to include them also in the agglutination tests at that time, but the previously prepared sera having been exhausted, it was necessary to bleed the animals again. This was done without further immunization and the agglutination content of the sera was found to be quite low; in several instances the homologous titrations even gave negative results at 1:20 (see table 3e). Notwithstanding, the Nevada strains gave positive results in every instance, and with the serum of rabbit 206 anti 1322, a definitely positive reaction at 1:5120. It is interesting to note that serum of rabbit 208 anti 1303, in spite of its low agglutinin content, was still quite antitoxic and was used successfully in the protection experiments at this time.

We also raised the question as to whether precipitins were involved in the reactions observed. Accordingly on two occasions tests were made using uninoculated glucose broth and bacteria-free filtrates of the forty-eight-hour homologous cultures as antigens. These tests were entirely negative.

The complete study involved fifteen different protocols. Table 4 illustrates one such protocol using serum rabbit 274 anti *Clostridium oedematoides* 1316.

TABLE 5

Summary of agglutination tests with anti B. Sordellii serums

ANTIGEN	SERA					
	Rabbit 261 Anti 1302	Rabbit 208 Anti 1303	Rabbit 274 Anti 1316	Rabbit 232 Anti 1317	Rabbit R19 Anti 1318	Rabbit 208 Anti 1322
B. Sordellii 1302	+ 1:2,560	++ 1:160	++ 1:160	++ 1:10,240	+ 1:5,120	++++ 1:10,240
B. Sordellii 1303	+ 1:10,240	+ 1:10,240	+ 1:5,120	++ 1:10,240	+ 1:10,240	+ 1:2,560
C. oedematoides 1316	++ 1:10,240	− 1:20	+ 1:320	+ 1:80	+ 1:40	+ 1:40
C. oedematoides 1317	++ 1:10,240	++ 1:40	+ 1:5,120	+ 1:2,560	+ 1:640	+ 1:5,120
C. oedematoides 1318	++ 1:10,240	± 1:20	+ 1:5,120	+ 1:5,120	+ 1:640	+ 1:5,120
B. Sordellii 1322	++ 1:10,240	+ 1:1,280	+ 1:10,240	+ 1:2,560	+ 1:10,240	+ 1:1,280
B. Sordellii* 2782	+ 1:160	+ 1:80	+ 1:2,560	+ 1:640	+ 1:5,120	+ 1:5,120
B. Sordellii* 2783	+ 1:2,560	± 1:20	+ 1:40	+ 1:320	+ 1:5,120	+ 1:5,120
B. sporogenes 10			− 1:20	+ 1:20	+ 1:20	+ 1:20
B. tyrosinogenes 106			− 1:20	− 1:20	+ 1:20	+ 1:20
B. Novyi 140			− 1:20	− 1:20	+ 1:20	+ 1:20
Bacteria-free filtrate†					− 1:20	− 1:20
Uninoculated dextrose broth					− 1:20	− 1:20

* These strains were tested separately with sera freshly drawn from the rabbits which had not received any immunization for several months and whose blood no longer contained strong homologous agglutinins, except in the case of rabbit R19 anti-1318. See table 3.

† Homologous cultures for serum.

Table 5 is a composite of all the titer limits obtained in the study.

The outstanding feature of table 5 is that it shows cross agglutination in all but one of the 48 tests conducted with *B. Sordellii* and *C. oedematoïdes*. Although the titers in some instances are somewhat low, the occurrence of cross agglutination in so high a percentage of tests confirms the serologic identity of the various strains, and suggests that agglutination tests with a potent serum, such as anti 1302, might be used to establish identity in the case of new strains which may be found. A negative result, however, could not be considered as establishing the non-identity of strains; witness the non-agglutination of strain 1316 by serum anti 1303.

Another feature is the general failure of cross agglutination with heterologous species. The positive results in low dilution in 3 out of 6 such check tests cannot be explained, but it must be noted that although weakly positive reactions did occur in low dilutions, such low titer limits were duplicated in only 3 out of the 48 tests with the homologous species, and these were in a serum of notably low titer for all heterologous strains. This circumstance, coupled with the fact that the heterologous species were agglutinated only slightly in the lowest dilution of sera which were relatively potent for the homologous species, justifies the conclusion that there is no real cross agglutination between these species.

The negative precipitin reaction is significant as showing that the animals had not become immunized to proteins in the broth. The objection might be raised here that the usual method of conducting a precipitation test is by using the serum undiluted or only slightly diluted, and making dilutions of the antigen, but it was felt that such a procedure in this case would not give results comparable to the agglutination reactions. As it was, we feel quite sure that precipitation played no part in the results observed.

It was noted that serum anti *B. Sordellii* 1302 agglutinated

all strains except the homologous strain to the highest dilution tested, while serum anti *B. Sordellii* 1303 clumped only the homologous strain to that extent. This is interesting because of the fact that the two are sibling strains, isolated from a single parent culture as "smooth" and "fluffy" colonies, although as Hall and Scott (1927) pointed out, both colonial types might be found in an old brain culture of either.

We found *C. oedematoides* 1316 to be somewhat refractory to cross agglutination, although it gave rise to a strong agglutinating serum; *B. Sordellii* 1303, on the other hand, was readily agglutinated, but produced a serum with a strong tendency toward strain specificity. Also, strain 1316 was agglutinated to a higher titer in sera against strain 1302 than in its own serum, while strains 1317 and 1318 were more susceptible to the action of serum against strain 1316 than to their own homologous sera. We cannot attempt to explain these inconsistencies, but it is possible that the observations of Weil and Felix, White, Gardner, Olitzky, Arkwright, Braun and others on multiple antigens, which led to the qualitative receptor analysis of Weil and Felix and to the work of Felix and Robertson (1928), may yield a solution of the problem.

<div align="center">CONCLUSIONS</div>

1. There is cross agglutination between all strains of *B. Sordellii* and *C. oedematoides*.

2. Sera prepared against *B. Sordellii* or *C. oedematoides* do not cross agglutinate *B. sporogenes*, *B. tyrosinogenes* or *B. Novyi*.

3. *B. Sordellii* and *C. oedematoides* are therefore distinct serologically from other species of obligately anaerobic bacilli.

Since the completion of our work Vawter and Records have reported in connection with their "Serologic Study of Sixteen Strains of Bacillus Hemolyticus" in the *Journal of Infectious Diseases* for July, 1931, that successful agglutinating serums were prepared by them against three strains of *B. Sordellii*, but these serums failed to agglutinate *B. hemolyticus*, also that there was no cross agglutinating of *B. Sordellii* by serums prepared with *B. hemolyticus*.

REFERENCES

BAUER, J. H., AND MEYER, K. F. 1926 Human intestinal carriers of tetanus spores in California. Jour. Inf. Dis., **38**, 295.

FELIX, A., AND ROBERTSON, M. 1928 Serological studies in the group of the spore bearing anaerobes. I. The qualitative analysis of the bacterial antigens of *B. septicus* and *B. tetani*. Brit. Jour. Exp. Path., **9**, 6.

HALL, I. C. 1922a The production of tyrosine by a putrefactive anaerobe. Absts. Bact., **6**, 6.

HALL, I. C. 1922b Differentiation and identification of the sporulating anaerobes. Jour. Inf. Dis., **30**, 445.

HALL, I. C. 1929 The occurrence of *Bacillus Sordellii* in icterohemoglobinuria of cattle in Nevada. Jour. Inf. Dis., **45**, 156.

HALL, I. C., RYMER, M. R., AND JUNGHERR, E. 1929 Comparative study of *B. sordellii* (Hall and Scott) and *Clostridium oedematoides* (Meleney, Humphreys and Carp). Jour. Inf. Dis., **45**, 42.

HALL, I. C., AND SCOTT, J. P. 1927 *Bacillus Sordellii*, a cause of malignant edema in man. Jour. Inf. Dis., **41**, 329.

HUMPHREYS, L. B., AND MELENEY, F. L. 1928 The identity of *C. oedematoides* and *B. Sordellii*. Proc. Soc. Exp. Biol. and Med., **25**, 611.

JUNGHERR, E. 1927 Unpublished manuscript.

SCHOENHOLZ, P., AND MEYER, K. F. 1923 Studies on the serologic classification of *B. botulinus*. I. Preparation of antiserum. Jour. Inf. Dis., **32**, 417.

SORDELLI, A. 1923 Flore anaerobie de Buenos Aires. Comptes rend. Soc. de Biol., **89**, 53.

TULLOCH, W. J. 1919 Report of bacteriological investigation of tetanus carried out on behalf of the war office committee for the study of tetanus. Jour. Hyg., **18**, 103.

WEINBERG, M., AND BAROTTE, J. 1929 Preparation des serums antigangreneux monovalents avec des toxines formolees. Ann. Inst. Past., **43**, 453.

THE LYSIS OF PNEUMOCOCCUS BY SAPONIN

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The solution of bacteria by bile (Neufeld (1900)), by fatty acids, and by the alkali soaps of these acids (Lamar (1911)) has led to a search for other reagents which would dissolve the bodies of bacteria. The lytic action of saponin on erythrocytes and other types of cells suggested the possibility of a similar action on the bacterial cell.

The term saponin is given to a group of plant glucosides. They are toxic for the higher animals as well as for trypanosomes (Neufeld and vonProwazek (1907)) and other protozoa (Bacon and Marshall (1906); Levaditi and Rosembaum (1905)), fungi (Boas, (1921); Goodwin, Salmon and Ware (1929)), filtrable viruses Landsteiner (1906); Allard (1918)), and spirochetes (Neufeld and von Prowazek (1907); Levaditi and Rosembaum (1908); Noguchi (1928)). Although the saponins may be regarded as general protoplasmic poisons the action on bacteria, in so far as it has been investigated, appears to be exceptional.

The mycobacteria alone have been found to be somewhat affected by saponin. Dostal (1916) reported that several types of the tubercle bacillus showed marked growth variation and loss of acid-fastness when grown on media containing 5 to 10 per cent saponin. Although at first denied (Schnurer (1923); Paltauf (1923)) these results have since been confirmed by other workers (Gildemeister (1923); Wyss (1923); Arima, Aoyama and Ohnawa (1924, 1930)). There is also the observation of Brooks (1921) that saponin causes a decrease in the rate of respiration of *B. subtilis*. As regards the action of saponin upon other bacteria, only negative results have been reported. Saponin has been found without

effect on air bacteria (Bacon and Marshall (1906)), *V. cholera, B. anthracis, B. typhosus, S. aureus, N. meningitidis,* (Landsteiner (1906); Russ (1906)), *B. Coli* (Rotky (1914)), and *Sp. giganteum* (Swellengrebel (1912)). Neufeld and Prowazek (1907) reported that sapotoxin had no destructive action on pneumococcus. Falk and Yang (1926) reported that pneumococcus types I, II and III were not dissolved by saponin.

The present work is a re-investigation of the lytic activity of saponin for pneumococcus. It was found that saponin does possess such activity, which is readily demonstrable under certain conditions.

The tests for saponin bacteriolysis were performed as follows: The pneumococci were tested in broth culture. The broth used was the standard beef infusion, adjusted to pH 7.8. Very early in the course of these experiments it was found a necessary precaution to avoid carrying over blood from the blood-broth stock cultures into the broths to be used in the tests. The tested culture was therefore always derived from a previous culture in plain broth. (The few exceptions to this procedure are noted below.) Aseptic technique was employed throughout. From the one-day broth cultures, 0.5 cc. was pipetted into each of three test tubes (100 × 13 mm.). To each was added either 0.5 cc. of a 10 per cent solution of saponin in saline (saponin Merck pure), 0.5 cc. saline, or 0.5 cc. of 10 per cent bile in saline. The saponin solution was usually freshly prepared, and was heated for fifteen minutes in boiling water before use. It was found that such heating does not affect the bacteriolytic activity. The tests were observed for two to three hours at room temperature, were then placed in the refrigerator and examined again the following day.

The development of complete transparency in the tube containing the saponin-treated bacteria was regarded as evidence of complete lysis. Lysis was often observed in a few minutes and was usually complete within thirty minutes.

To test for dissolution of the bacterial bodies, a loopful of the saponin-treated suspension was emulsified in a loopful of Loeffler's methylene blue on a cover slip, and examined microscopically in the hanging drop (Mandelbaum (1907)). It was found that

saponin does not prevent the staining of pneumococci but that it does prevent fixation of the organisms to a glass slide, so that the ordinary heat-fixed preparations could not be used. In the case of complete lysis, hanging drop preparations made after twenty-four hours usually revealed no intact bacteria. It was evident, however, that viable pneumococci remained, since inoculation of the entire 1 cc. quantity of the dissolved culture into broth always yielded a growth of pneumococci. A few plate counts were made; these indicated a very marked reduction in the number of viable organisms when lysis had occurred. No such reduction was evident in the case of pneumococci which were not dissolved after treatment with saponin.

Our first observation was made on a virulent strain of pneumococcus type I, which had undergone a series of rabbit passages. The bacteria were completely dissolved in 5 per cent saponin. A culture strain of the same type was not affected. It therefore seemed necessary to determine whether saponin solubility is correlated with either virulence or animal passage.

Strains of pneumococcus[1] types I, II, etc., to VIII were submitted to mouse passage. Mice were inoculated intraperitoneally with 0.5 cc. of the eighteen hour broth cultures. After death of the experimental animal the heart's blood was inoculated into plain broth. On the following day these cultures were tested for lysis by saponin. In the case of several strains, the mouse-passage culture exhibited complete solubility. Repetition of the experiment, however, did not give constant results.

It was evident, therefore, that other factors than animal passage play a rôle in the lysis by saponin. A possible factor appeared to be the presence of blood in the culture medium. The mouse-passage culture which had shown lysis contained a small amount of mouse blood. In order to determine whether lysis is connected with the presence of blood, the strains which had manifested lysis after mouse passage were inoculated into plain broth and into blood broth (2 drops sterile sheep blood in about 7 cc. broth). In each case a plain broth culture was used as inoculum. On

[1] These strains were obtained from the Bureau of Laboratories, Department of Health, New York City.

testing for lysis the pneumococci in the blood broth cultures proved to be soluble, while those in plain broth were insoluble. It was thus seen that solubility is related to the presence of blood in the culture. That this factor is responsible for the lysis observed after animal passage was further indicated by the following observation: of two pneumococcus cultures taken simultaneously from the same infected mouse, only one proved to be saponin-soluble; however, subcultures from both manifested solubility, depending on the presence or absence of blood in the culture medium: subcultures in blood broth showed lysis, while those in plain broth did not.

In addition to whole blood, it was found that plasma, serum, and ascitic and pleural fluids rendered the pneumococcus soluble when present in the broth in which the organism was cultivated. Thirteen strains of pneumococcus were investigated in this connection. All were saponin-insoluble when tested in plain broth culture. Nine (including two "rough" strains) were soluble when grown in broth containing blood or other animal fluid. The remaining four strains were not reactive in this sense to the presence of animal fluid. We are not at present able to account for the difference between "reactive" and "non-reactive" strains. In the following experiments (except where a modifying statement is made) "reactive" strains of types III, IV and VIII were always used.

The substance in these animal fluids which is responsible for the development of saponin solubility was found to be thermostable when heated to 56°C. for thirty minutes. Pneumococci grown in heated blood and ascitic broth were found susceptible to lysis by saponin.

The minimum concentration of serum which is necessary for saponin lysis was determined by titration as shown in table 1 which shows that the extent and rapidity of lysis vary directly with the amount of serum present in the medium.

The pneumococcus does not retain the saponin solubility which it presents in mediums containing blood or other animal fluid, after inoculation into plain broth; although lysis was occasionally observed in the first subculture it was not shown by the second

TABLE 1

Saponin lysis of pneumococcus (type III) grown in broth containing varying concentrations of serum

TIME OF INCUBATION	SHEEP SERUM (IN 5 CC. BROTH) AMOUNTS					
	0.5 cc.	0.1 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0 cc.
15 minutes	Almost complete	Partial	Slight	No lysis	No lysis	No lysis
30 minutes	Complete	Almost complete	Slight	No lysis	No lysis	No lysis
120 minutes	Complete	Almost complete	Partial	No lysis	No lysis	No lysis
After 20 hours in refrigerator	Complete	Complete	Almost complete	Partial	No lysis	No lysis

TABLE 2

Saponin solubility of pneumococci grown in broth containing varying concentrations of cholesterol

Cholesterol (in 5 cc. broth) amounts

1.0 mgm.	10^{-1} mgm.	10^{-1} mgm.	10^{-3} mgm.	10^{-1} mgm.	10^{-4} mgm.	10^{-5} mgm.	10^{-6} mgm.	0 mgm.
Complete	Complete	Complete	Usually almost complete*	Slight or partial*	No lysis	No lysis	No lysis	No lysis

Alcoholic solutions of cholesterol were added to sterile test tubes. After evaporation of the alcohol, sterile broth was added to the solid residue. The cholesterol broth was then incubated for twenty-four hours to insure sterility. Although cholesterol is "insoluble" in broth it is very probable that the minute amounts required, i.e. 10^{-6} grams, do go into solution.

* Usually evident only in later readings; different strains showed variations.

or later culture generations. Apparently the lysis requires a definite amount of body fluid in the culture medium. It was found, however, that the body fluid which is present free in the culture is not essential to lysis. Repeated washing of the bacteria to remove this fluid does not prevent complete lysis. It appears, therefore, that the body fluid, in producing susceptibility to saponin, effects some change in the bacterial cell itself. That this change is due to a direct action on the bacteria is shown by the following experiments: Pneumococci grown in plain broth were found to be saponin-insoluble. To the culture of each strain was added 0.5 cc. of sterile ascitic fluid. Portions of the mixtures were immediately tested and were found insoluble. The mixtures were incubated at 37°C. and tested again at half-hour intervals. After two or three hours' incubation the bacteria had become completely soluble. In order to exclude the possibility that reproduction of the bacteria is necessary for this development of saponin-solubility, the experiment was repeated under conditions which did not permit growth. Conversion to the saponin-soluble state was effected at a temperature of approximately 0°C. after about twenty-four hours. The controls without ascitic fluid remained insoluble.

It is apparent then that a constituent of the blood or ascitic fluid brings about a sort of sensitization of the bacterial cells by which they become susceptible to lysis by saponin.

We have mentioned above that addition of saponin immediately after the addition of ascitic fluid does not produce lysis. Furthermore, lysis does not appear even after prolonged incubation of the mixture. The bacteria must remain in contact with the ascitic fluid for a certain period of time *before* the saponin is added, in order that lysis may occur. The absence of lysis which is observed under the former conditions must be attributed to a failure of sensitization. These facts suggested the possibility that the saponin reacts with the sensitizing constituent of the ascitic fluid and renders it inactive. Consideration of this possibility afforded a clue as to the identity of the sensitizing substance. The animal fluids which had been found to possess sensitizing activity contain cholesterol. Saponin has a strong affinity for cholesterol and

combines with it to form the physiologically inactive saponin-cholesteride (Windaus (1909); Yagi (1910–11)). If cholesterol is the active constituent of these fluids its combination with saponin might explain the failure to sensitize when saponin is present. We attempted, therefore, to determine whether the sensitizing activity of body fluid is due to its cholesterol content, by tests of the sensitizing activity of cholesterol alone and by fractionation of ascitic fluid with tests of the activity of the separate fractions.

The sensitizing activity of cholesterol was determined by testing the saponin-solubility of pneumococci grown in broth to which had been added a varying amount of cholesterol (chemically pure, Eimer and Amend). Table 2 gives a summary of the results and shows that under the experimental conditions 0.01 mgm. cholesterol always suffices to produce complete solubility. Sheep serum, according to Kauders (1913), contains 0.87+ mgm. free cholesterol per cubic centimeter. It is evident, therefore, that the cholesterol content is sufficiently high to account for the sensitizing activity of sheep serum (cf. table 1).

It was also found possible to sensitize by means of cholesterol, plain broth pneumococcus cultures at temperatures of 37° and 0°C., with results essentially similar to those obtained with body fluid. It should be stated that the time required for lysis in these experiments was not constant, but it was always evident that the extent and rapidity of lysis increases with the duration of the sensitization period. In general, lysis is slower when the grown culture is sensitized than when the cholesterol or body fluid is added to the medium in which the organism is cultivated.

The fractionation of ascitic fluid was performed as follows:

Ascitic fluid was extracted with 6 volumes of ether by four days contact in a rotating flask. The concentrated ether extract was treated with acetone to precipitate the lecithin fraction. (Only a negligible amount of precipitate was obtained.) To remove the fatty acids, the ether-acetone soluble fraction was evaporated to dryness, saponified with excess NaOH, re-evaporated to dryness, and extracted with anyhdrous ether. The extract gave a positive Liebermann-Burchard test for cholesterol.

In testing for the activity of the several fractions a sample of

the dry material was suspended in saline, sterilized in boiling water, and added aseptically to tubes of plain broth. In the case of the extracted ascitic-fluid fraction 0.5 cc. of the sterile fluid was added to the broth directly. The saponin solubility of type III and type IV strains grown in these broths served as index of the activity of the test substance.

TABLE 3

Fractionation of ascitic fluid

```
                      Ascitic fluid
                      ‾‾‾‾‾‾‾‾‾‾‾‾‾(active)
                               | ether extraction
          ┌────────────────────┴────────┐
   Extracted ascitic fluid          Ether  extract
   ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾           ‾‾‾‾‾‾‾‾‾‾‾‾‾
         (inactive)                    (active)
                                          | acetone precipitation
               ┌──────────────────────────┴──────┐
        Lecithin fraction          Ether-acetone  filtrate
        ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾            ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
       (insufficient yield)                (active)
                                           | saponification and
                                           | ether extraction
          ┌────────────────────────────────┴─┐
   Soap fraction          Cholesterol fraction
   ‾‾‾‾‾‾‾‾‾‾‾‾‾           ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
   (doubtful activity)*          (active)
```
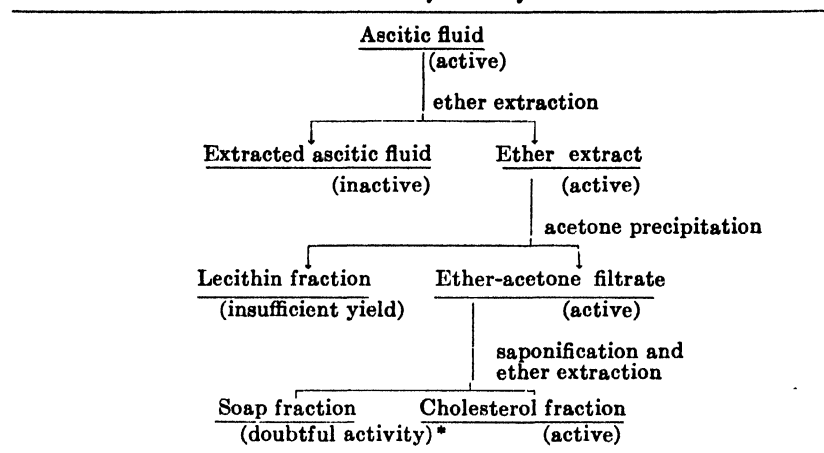
*The soap fraction was growth inhibitory in high concentration. Smaller concentration showed some degree of activity. It is doubtful whether the mechanism of this activity is identical with that of cholesterol. Lamar (1911) has shown that soaps are in themselves actively lytic for pneumococci and, even in high dilution, appear to alter the integrity of the bacterial membrane.

The experiment is summarized in table 3 which shows that the active constituent of ascitic fluid is found in the cholesterol fraction.

The failure to obtain a lecithin fraction from ascitic fluid necessitated the use of a preparation of this substance from another source. The cholesterol was removed from a lecithin preparation (lecithin from eggs; Eimer and Amend) by acetone precipitation of an ethereal solution of the commercial product. The filtrate containing cholesterol was tested as above and found active. The precipitate which contained lecithin without cholesterol proved to be inactive.

It was of interest to test for the activity of a cholesterol ester for two reasons: (1) a certain percentage of esterified cholesterol occurs in animal fluids; (2) cholesterol esters in contrast to cholesterol have no affinity for saponin (Hausmann (1905); Abderhalden and LeCount (1906)). Cholesteryl acetate (Eastman Kodak Company) tested by the method employed in the titration of cholesterol proved to be inactive in concentrations of from 0.1 to 10.0 mgm. per 5 cc. The ester was then converted to cholesterol by hydrolysis with alcoholic NaOH. The recovered cholesterol was found active in 0.1 and 0.01 mgm. amounts and partially active in 0.001 mgm. amount. The fact that two different cholesterol preparations show the same activity titer (cf. table 2) affords evidence that we are not concerned with an impurity associated with cholesterol.

The hemolytic action of saponin is known to be inhibited by cholesterol but not by cholesterol esters (Hausmann (1905); Abderhalden and LeCount (1906)). It was of interest to determine if the same is true in the case of saponin bacteriolysis. This necessitated a determination of the minimum active concentration of saponin. Both problems were investigated as shown by the following experiment:

To eliminate the complicating factor of broth the bacteria were tested in saline suspension. Pneumococci grown in 0.002 per cent cholesterol broth were collected by centrifugation and suspended in sufficient saline to yield the same opacity as a *S. aureus* suspension containing approximately 10^9 organisms per cubic centimeter. Amounts of 0.5 cc. of the pneumococcus suspensions were tested for lysis by 0.5 cc. of each of the following reagents: (1) increasing dilutions of saponin in saline; (2) filtrates of saponin solutions which had been treated with varying amounts of cholesterol or cholesteryl acetate, following in the main the method of Kofler and Schrutka (1925); (3) reagents prepared as in (2), omitting, however, the filtration.

The results are summarized in table 4 which shows: (1) saponin Merck is actively bacteriolytic in 1:20,000 dilution (0.05 mgm. saponin per cubic centimeter); (2) a completely inactive filtrate is obtained on addition of 0.25 mgm. cholesterol to a

TABLE 4

Saponin lysis of pneumococci grown in cholesterol broth and inhibition of lysis by cholesterol

0.5 CC. OF PNEUMOCOCCUS SUSPENSIONS + THE FOLLOWING REAGENTS TO MAKE A TOTAL VOLUME OF 1.0 CC.

PNEUMO-COCCUS TYPE	Saponin					Filtrate from		0.5 mgm. saponin +	
	0.5 mgm.	0.1 mgm.	0.05 mgm.	0.025 mgm.	0.01 mgm.	0.5 mgm. saponin + 0.25 mgm. cholesterol	0.5 mgm. saponin + 0.5 mgm. cholesteryl acetate	0.5 mgm. cholesterol	0.5 mgm. cholesteryl acetate
I	Complete	Complete	Almost complete	No lysis	No lysis	No lysis	Complete	(4)*	Almost complete†
III	Complete	Complete	Almost complete	No lysis	No lysis	No lysis	Complete	(10)*	Almost complete
VIII	Complete	Complete	Almost complete	No lysis	No lysis	No lysis	Complete	(10)*	Almost complete

The macroscopic readings were taken after two hours at room temperature. The tests were then placed in the refrigerator and examined again the following day. Lysis had occurred to a varying degree of completeness in the 0.025 mgm. saponin column; the lysis had become complete in the case of type VIII tested in 0.05 mgm. of saponin. All the other tests appeared unchanged.

* The precipitate formed by saponin + cholesterol allowed only microscopic determination of lysis. The recorded numerals are rough approximations of the comparative number of organisms present after the refrigeration period, the number in completely undissolved suspensions being considered as 10. A decrease in these comparative numbers was observed after further refrigeration (two and three days). This partial lysis may be due to the saponin slowly liberated from the saponin-cholesterol complex (cf. Madsen and Noguchi (1905); Reisenfeld and Lummerzheim (1913)).

† The reagent used in these tests contained crystals of cholesteryl acetate, but the fluid itself was clear. Obviously, a macroscopic reading of "complete" was not to be expected. No organisms were found on microscopic examination.

solution containing 0.5 mgm. saponin; cholesteryl acetate does not remove saponin from solution; (3) saponin bacteriolysis is inhibited by cholesterol but not by cholesteryl acetate. The inhibition is apparently due to the fact that there is formed an insoluble saponin—cholesteride.

The inhibition of saponin by cholesterol finds its converse in the present study: the inhibition of cholesterol by saponin. It has been noted that saponin inhibits sensitization by body fluid. It was suggested that this inhibition is due to a combination of saponin with the cholesterol of the body fluid. It was found, furthermore, that a like inhibition is obtained when sensitization is undertaken with cholesterol. In order to confirm the suggested explanation, it was necessary to determine whether cholesterol loses its sensitizing activity when bound by saponin. This was attempted as follows: To saponin-cholesterol mixtures prepared by incubation of 0.5 mgm. saponin with 0.01 mgm. cholesterol were added saline suspensions of pneumococci derived from plain broth cultures. The suspensions were then incubated at room temperature and tested at intervals for solubility in 5 per cent saponin. It was found that: (1) sensitization did occur to a degree which varied with the several strains from slight to almost complete; (2) as compared with controls treated with cholesterol alone, the time required for sensitization was greater, and the degree of sensitization in equal time intervals was less. We concluded that cholesterol suffers a diminution of sensitizing activity when bound by saponin. Since this union is unstable (Yagi (1910–11); Madsen and Noguchi (1905); Reisenfeld and Lummerzheim (1913)), it was to be expected that some degree of sensitization would occur due to the cholesterol slowly liberated. That sensitization was completely inhibited in the experiments referred to above is very probably due to the fact that in these cases the concentration of saponin was some thousand times the concentration of cholesterol. This considerable excess of saponin would retard the liberation of cholesterol from the complex.

It may be noted that eight strains of streptococci tested in plain, cholesterol, and serum broth culture were all found 'to be insoluble in 5 per cent saponin.

DISCUSSION

The toxicity of saponin for various cells, e.g. erythrocytes (Ransom (1901)), trypanosomes (Neufeld and vonProwazek (1907)), paramecia (Levaditi and Rosembaum (1908)), has been attributed to its affinity for the cholesterol of the cell. This theory is largely based on Ransom's (1901) findings in the study of saponin hemolysis. He discovered the affinity which exists between saponin and the erythrocyte cholesterol, and concluded that hemolysis by saponin is due to an "attack" on the cholesterol. The numerous subsequent investigations have not led to complete acceptance of this interpretation. It is beyond the scope of the present discussion to enter into the controversial literature, for which reference is made to the recent monograph by Kofler (1927). It suffices to note here that in no instance has it been demonstrated that a union between saponin and cell-cholesterol is the cause of the disruption of the cell. The present study, on the other hand, affords evidence in favor of this explanation of saponin action.

It has been shown that pneumococci, although normally resistant, are made susceptible to saponin by treatment with cholesterol. The experimental findings favor the interpretation of the resulting bacteriolysis in terms of a union between saponin and the cholesterol which may have been adsorbed or otherwise assimilated by the bacterial cell during the sensitization with cholesterol. We have seen that the sensitization process is not dependent on a modification of bacterial growth but involves an action on the bacteria directly. The sensitized bacteria retain their saponin-susceptibility even after several washings. That adsorption may participate in this sensitization gains credence from the work of Eisler (1926) which shows that cholesterol adsorbed on bone-black resists removal by washing and retains its ability to bind saponin. The importance of the saponin-cholesterol union in the lysis reaction is emphasized by the observation that when cholesterol is esterified it loses its affinity for saponin, and at the same time becomes completely ineffective in sensitizing pneumococci to saponin. It appears, furthermore, that the union be-

tween saponin and cholesterol must take place on the bacterial cell in order to effect lysis.   This condition may be formulated as follows: when saponin unites with cholesterol before the latter is affixed to the cell, sensitization is inhibited and no lysis occurs; when the saponin unites with cholesterol already present on the cell, lysis results; when cholesterol is present in excess in the surrounding fluid, lysis is again inhibited, since this excess cholesterol combines with the saponin, thus diverting it from the sensitized cell.

The saponin lysis of cholesterolized pneumococci affords, in so far as we know, the only example of the toxic action of saponin on any of the true bacteria.[2]   The insusceptibility of bacteria to this protoplasmic poison has been attributed to the presence of a resistant cell membrane (Bacon and Marshall (1906); Swellengrebel (1912)).   It is possible that in the present instance, the union of saponin with the cholesterol on the cell causes a disruption of the cell membrane, thus allowing further destruction and, finally, complete dissolution of the pneumococcus cell.

It is interesting to note the parallelism disclosed between saponin hemolysis and saponin bacteriolysis.   Cholesterol is seen to play an important rôle in both reactions.   Furthermore, the bacteriolytic activity of saponin Merck approximates its hemolytic activity (Kofler and Lazar (1927)).   Both activities are destroyed by comparable concentrations of cholesterol (Hausmann (1905); Kofler and Schrutka (1925); Ransom (1901)), and are not affected by cholesteryl acetate (Hausmann (1905); Abderhalden and Le Count (1906)).

[2] Perles (1890) reported that solanine inhibited bacterial growth.   However, solanine can hardly be considered a true saponin since it contains nitrogen.

Since the present study has been completed, Downie, Stent and White (Brit. Jour. Exp. Path., 1931, xii, 1) have recorded the observation that a strain of pneumococcus (group IV) in hormone broth culture was completely lysed by sapotoxin in dilutions of from 1:20 to 1:800.   No further details were given.   Evaluation of this observation is impossible in the lack of information as to the source of the sapotoxin, the cholesterol content of hormone broth, and the amount of blood carried over in the inoculum.

SUMMARY

1. Pneumococci are not dissolved by saponin when tested in plain broth culture. Treatment of the bacteria with cholesterol renders them susceptible to complete and rapid lysis by saponin.

2. The cholesterol exerts a direct action on the bacteria, independent of bacterial reproduction.

3. The bacteria must be in contact with the cholesterol for a definite period of time prior to the addition of saponin in order to obtain lysis. Excess of saponin inhibits the sensitization by cholesterol. Conversely, excess of cholesterol inhibits lysis by saponin.

4. Animal fluids, e.g. blood, or ascitic and pleural fluids, act similarly to cholesterol. Evidence is presented to show that the activity of these fluids is due to their cholesterol content.

5. When cholesterol is esterified it loses its affinity for saponin, and becomes incapable of sensitizing pneumococci to saponin.

6. The theory is advanced that the lysis is due to a union of saponin with the cholesterol assimilated by the bacteria during sensitization.

7. The parallelism between saponin bacteriolysis and saponin hemolysis is noted.

REFERENCES

ABDERHALDEN, E., AND LeCOUNT, E. R. 1906 Z. exp. Path. u. Therap., **2**, 199.
ALLARD, H. A. 1918 Jour. Agric. Res., **13**, 634.
ARIMA, R., AOYAMA, K., AND OHNAWA, J. 1924 Deutsch. med. Woch., **50**, 660.
ARIMA, R., AOYAMA, K., AND OHNAWA, J. 1930 Seuchenbekämpfung Infektionskrankh., **7**, 225.
BACON, R. F., AND MARSHALL, H. T. 1906 Philipp. Jour. Sci., **1**, 1037.
BOAS, F., 1921 Biochem. Z., **117**, 166.
BROOKS, M. M. 1921 Jour. Gen. Physiol., **3**, 531.
DOSTAL, H. 1916 Frank. Z. Path., **19**, 198.
EISLER, M. 1926 Biochem. Z., **172**, 154.
FALK, I. S., AND YANG, S. Y. 1926 Jour. Inf. Dis., **38**, 8.
GILDEMEISTER, E. 1923 Centr. Bakt., 1 Abt., Orig., **89**, 154.
GOODWIN, W., SALMON, E. S., AND WARE, W. M. 1929 Jour. Agric. Sci., **19**, 185.
HAUSMANN, W. 1905 Beitr. chem. Physiol. u. Path., **6**, 567.
KAUDERS, F. 1913 Biochem. Z., **55**, 96.
KOFLER, L. 1927 Die Saponine. Julius Springer, Wien.
KOFLER, L., AND LAZAR, Z. 1927 Wien klin. Woch., **40**, 16.
KOFLER, L., AND SCHRUTKA, W. 1925 Biochem. Z., **159**, 327.

LAMAR, R. V. 1911 Jour. Exp. Med., **13**, 1, 256.

LANDSTEINER, K. 1906 Centr. Bakt., 1 Abt., Ref., **38**, 542.

LEVADITI, G., AND ROSEMBAUM, H. 1908 Ann. Inst. Pasteur, **22**, 323.

MADSEN, T., AND NOGUCHI, H. 1905 Chem. Centr., **1**, 1265.

MANDELBAUM, M. 1907 Münch. med. Woch., **54**, 1431.

NEUFELD, F. 1900 Z. Hyg., **34**, 454.

NEUFELD, F., AND VONPROWAZEK, S. 1907 Arb. k. Gesundhtsamte., **25**, 494.

NOGUCHI, H. 1928 In Jordan and Falk, Newer Knowledge of Bacteriology and Immunology, Chicago.

PALTAUF, R. 1923 Centr. Bakt., 1 Abt., Orig., **89**, 154.

PERLES, M. 1890 Arch. exp. Path. u. Pharm., **26**, 88.

RANSOM, F. 1901 Deutsch. med. Woch., **27**, 194.

REISENFELD, E. H., AND LUMMERZHEIM, H. 1913 Z. physiol. Chem., **87**, 270.

ROTKY, K. 1914 Centr. Bakt., 1 Abt., Orig., **73**, 195.

RUSS, V. K. 1906 Arch. Hyg., **59**, 302.

SCHNURER 1923 Centr. Bakt., 1 Abt., Orig., **89**, 150.

SWELLENGREBEL, N. H. 1912 Centr. Bakt., 1 Abt., Ref., **51**, 146.

WINDAUS, A. 1909 Ber. chem. Ges., **42**, 1, 238.

WYSS, F. 1923 Compt. rend. Acad., **177**, 719.

YAGI, S. 1910–11 Arch. exp. Path. u. Pharm., **64**, 141.

THE EFFICIENCY OF CERTAIN GERMICIDES IN THE PRESERVATION OF BIOLOGIC PRODUCTS[1]

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The matter of preservation is of great importance in the manufacture of biologic products that are to be employed in the prevention and treatment of disease.   Phenol and Trikresol are generally used for this purpose, but they are not entirely satisfactory. With the comparatively recent development of a number of new germicidal substances, it seemed appropriate to make a study of some of them to determine whether or not they might be employed to advantage.

There are five features of major importance which must be known in order to judge the potentialities of a germicide as a biologic preservative.   These are:

1. The germicidal action upon spores and vegetative cells of bacteria in different media.
2. The effect upon the antigenic or immunizing value of the various products.
3. Effect upon the appearance of the product and the precipitation of protein or other substances.
4. Toxicity of the germicide, with special reference to the margin of safety as measured by intravenous, intramuscular, and intraspinal toxicity tests.
5. The histologic changes which the germicide produces in animal tissues.

The problem was divided into two parts with different methods of approach.   The first part was a study of the germicidal power

[1] A part of a thesis submitted to the Faculty of the Graduate School of the University of Maryland in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

of the various chemical substances, together with the physical effect upon the basic media employed. Only substances found satisfactory in the first phase of the problem were given further study. The second part of the work was a study of the toxicity of the preservative, its effect upon the appearance and potency of the biologic products, and the histologic changes it produces in the tissues of experimental animals.

The following germicides were chosen:

1. Phenol (crystals) U. S. P.
2. Hexylresorcinol (S.T. 37) (1:1,000)
3. Colloidal chloro-cresol (25 per cent)
4. Colloidal chloro-thymol (25 per cent)
5. Colloidal chloro-cresol (50 per cent)
6. Colloidal chloro-thymol (50 per cent)
7. Trikresol
8. Yatren (powder)
9. Mercurophen (2 per cent aqueous solution)
10. Metaphen (2 per cent in 2 per cent NaOH solution)

I. GERMICIDAL TESTS. DETERMINATION OF GERMICIDAL POWER IN SALT SOLUTION, BOUILLON, AND SERUM, TOGETHER WITH COL-ORATION AND PRECIPITATION OF PROTEIN SUBSTANCES

As menstruums, sterile isotonic salt solution and plain bouillon made from beef infusion to which 1 per cent peptone and 0.5 per cent sodium chloride had been added were used. The reaction was adjusted to a pH value of 6.8. The medium was checked for sterility after having been autoclaved for one-half hour at 15 pounds pressure. Sterile horse serum was also employed.

Method of making dilutions. The dilutions were made so that the total volume of chemical and medium was 5 cc. The germicide was added directly to the medium without previous dilution with distilled water, because it was found in earlier experiments that otherwise the medium was diluted to such an extent that it no longer supported growth.

Organisms employed. Four bacterial species were employed in each dilution of the germicide; namely, *Escherichia coli, Staphylococcus albus, Bacillus mycoides,* and *Bacillus subtilis,* because it

has been demonstrated that many germicides are more effective against some species of bacteria than others. The first two species produce vegetative cells only and the latter two are spore-forming bacteria.

Inoculations and controls. One-tenth of a cubic centimeter of each of the cultures was added to 10 cc. plain broth and the whole incubated for twenty-four hours at 37.5°C. At the expiration of this period the cultures were filtered through sterile filter paper to remove the clumps and to produce as uniform a suspension of the bacteria as possible. One-tenth of a cubic centimeter of each of these cultures was added to the specific dilutions of the germicide, using one tube for each dilution for each of the four organisms. After inoculating the various dilutions, they were allowed to stand at room temperature for twenty-four hours.

Determining the germicidal action of the chemicals. The tubes were next examined for visible growth and the results were recorded. Then each dilution was plated separately on plain agar. One cubic centimeter of each of the dilutions was seeded over the surface of plates that had previously been poured and incubated for sterility. The inoculated plates were then placed at 37°C. for twenty-four hours and examined for bacterial growth.

Physical effect of the germicides upon the medium. Observations were made (*a*) upon the coloring action of each germicide on the various media employed, (*b*) the appearance of turbidity in the salt solution, (*c*) precipitation of protein or other material in the bouillon and serum and (*d*) miscibility of the germicide with the medium.

Study of germicidal action

Phenol, due to its antiseptic properties, has long been employed as a preservative for biologic products. Phenol, 1:333 parts in isotonic salt solution, 1:200 parts in bouillon, and 1:200 parts in serum killed all four species of bacteria within the twenty-four hour observation period and caused no discoloration of the salt solution, bouillon, or serum. There was precipitation in the bouillon and serum at the dilutions in which it was germicidal. This precipitate formed almost immediately and increased with

agitation. In a comparatively short time it settled to the bottom of the tube leaving the medium practically clear. Phenol was also found to be miscible with the media at the dilutions employed.

Trikresol (ortho-, meta- and para-cresol). Trikresol gave a clear solution in aqueous 0.9 per cent sodium chloride. It caused no discoloration of the serum or bouillon, but, in the germicidal dilutions, gave a precipitate in both bouillon and serum. Bacteria were killed in salt solution containing Trikresol in a dilution of 1:500, in Trikresol-bouillon 1:333 parts, and in Trikresol-serum 1:250 parts.

Colloidal chloro-thymol (25 per cent). This is an amber-yellow solution, water-soluble, and with a weak thymol odor. Colloidal chloro-thymol is a chlorinated methyl-isopropyl-phenol of the formula $C_6H_2OH(1)CH_3(3)Cl(4)C_3H_7(6)$. In this preparation, by a substitution of the hydrogen atom, the halogen chlorine is introduced into the benzene nucleus. Graphically it is:

$$CH_3$$
$$Cl \overset{\displaystyle\bigcirc}{} OH$$
$$CH$$
$$CH_3 \quad CH_3$$
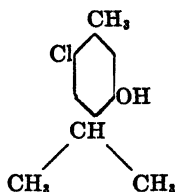
It was expected that the introduction of a halogen into the benzene nucleus would decrease its toxicity, and would thereby correspondingly increase its germicidal value (Laubenheimer, 1928).

This substance was miscible with the medium, gave the salt solution a cloudy, bluish-white appearance, and was effective in a dilution of 1:333. In a dilution of 1:200 in bouillon and serum, it killed all four test organisms. In both there was marked turbidity with heavy precipitation.

Colloidal chloro-cresol (25 per cent). This cresol derivative, also, is an amber-yellow solution, water-soluble, with a weak odor of cresol. In this preparation, as with colloidal chloro-thymol, the hydrogen atom is substituted by the halogen, chlorine.

Colloidal chloro-cresol (25 per cent) in salt solution was effective in a final dilution of 1:500, giving a pale pearl-gray color to

the solution. It was effective in a dilution of 1:333 in serum and 1:250 in bouillon. In the bouillon and serum there was heavy precipitation, with a rapid settling of the precipitate.

Colloidal chloro-cresol (50 per cent). *Colloidal chloro-thymol* (50 per cent). This strength of these substances was recommended by the manufacturer after failure of the 25 per cent solution to prove satisfactory in this work, due to the heavy precipitation of the protein material. Dilutions were made in salt solution, bouillon and serum from 1 per cent to 0.1 per cent. Although there was an increase in the germicidal action over the 25 per cent colloidal chloro-cresol and chloro-thymol, there was also increased precipitation.

Ether as a menstruum of preservatives for biologic products is effective for phenol and Trikresol in preventing precipitation, and so was added to the above colloids in equal parts in both the 25 and the 50 per cent strengths.

The results in the 50 per cent concentration were the same as those in the 25 per cent concentration, except in the case of the chloro-thymol. Here, in a concentration of 0.4 per cent it gave a heavy precipitate, and a slight precipitate in 0.2 per cent in broth. In a strength of 0.1 per cent a precipitate occurred in the serum.

The chloro-cresol in 0.3 per cent concentration precipitated bouillon and serum slightly. The results obtained in the precipitation of the protein materials, by colloidal chloro-cresol and colloidal chloro-thymol in both the 25 and 50 per cent strengths showed that ether would not prevent precipitation to an extent that would make their employment practicable.

Yatren. This is a German preparation, the chemical nature of which was not known by the author. It is a brown powder having a maximum solubility in water of 4 per cent. It is not germicidal in a 1:50 dilution. The media were colored from a deep brown to a light amber depending upon the dilution. In a 4 per cent solution there was no precipitation in bouillon or serum.

Hexylresorcinol (S.T. 37) (1:1,000). In a 1:1,000 dilution the solution is water clear. This substance has the formula $C_6H_3(OH)_2(C_6H_{13})$[2] and was miscible with the media. In a con-

[2] New and Non-official Remedies, 1929.

centration of 1:50 it was not germicidal in any of the basic media. It caused no discoloration and no precipitation of the bouillon or serum.

Metaphen (2 per cent in a 2 per cent NaOH solution). Metaphen, a proprietary preparation, known as 4-nitro-3, 5 bisacetoxymercuri-2-cresol (Raiziss and Sevarac, 1923). Its structural formula is:

$$CH_3CO-O-Hg \quad \overset{\displaystyle OH}{\underset{\displaystyle NO_2}{\bigcirc}} \overset{CH_3}{\underset{}{}} HgOOC\ CH_3$$
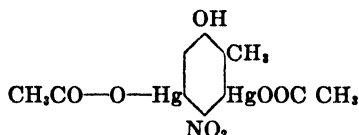
The 2 per cent solution is amber colored and it mixes readily with the media employed. Because of its high germicidal value, it was necessary to make an initial dilution of 1:1,000, using distilled water, before preparing the desired dilutions with the media.

Metaphen gave the media a faint amber colored appearance, but one that was not easily discernible. In a concentration as high as 1:200 there was no precipitation in either the bouillon or serum. It was germicidal in salt solution in a dilution of 1:10,000, in 1:8,333 parts in bouillon, and in 1:5,000 parts in serum.

For further study of the germicidal action of this substance, a number of bottles were prepared, some containing serum, and some bouillon. Metaphen, 1:8,000 in bouillon and 1:5,000 in serum was employed. Each was inoculated with about 0.5 gram of dust particles. These cultures showed no growth when in direct sunlight for several weeks, after which time there appeared in the serum a growth of air bacteria. At the end of six weeks the bouillon showed the presence of molds and various organisms. Metaphen in a 1:8,000 concentration was added to 100 cc. of tetanus toxin and was placed in each of five bottles. Also five bottles containing a similar amount of diphtheria toxin with Metaphen in a 1:8,000 dilution were inoculated with dust and incubated for two weeks at 37°C. At the end of this time they were examined and none showed growth. From these, five samples were placed in the sunlight and five in the dark. At the end of a month all five samples kept from the sun rays were sterile

while three of the five samples exposed to the action of sunlight showed growth. While no definite conclusions are drawn as to the effect of sunlight in causing a loss of the germicidal properties of Metaphen, it seems to have some apparent destructive action.

Mercurophen (2 per cent solution). Mercurophen, a proprietary preparation, is sodium-parahydroxymercuri-ortho-nitro phenolate. Structurally it is as follows (Raiziss and Sevarac, 1923):
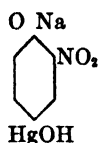
The 2 per cent aqueous solution is odorless with a brick-red color while in higher dilutions it is amber. In preparation for the germicidal tests, due to its ability to kill bacteria in high dilutions, a preliminary dilution of 1:1,000 in distilled water was made before completing the final dilutions by the addition of the basic media.

This substance was miscible with the media, and was germicidal in salt solution in a dilution of 1:16,500; bouillon, 1:12,500; and serum, 1:8,500. It imparts an amber hue not unlike that of normal serum and bouillon, but slightly more intense. There was no precipitation in serum in dilutions as low as 1:200.

A series of preparations were made, as in the case of Metaphen, under identical conditions, and exposed to sunlight to ascertain if there was loss of germicidal power. Bouillon containing Mercurophen in 1:12,000 parts, and serum 1:8,000 parts were prepared. After four months' exposure at room temperature the serum and bouillon were found to be sterile. After inoculating five samples of tetanus toxin, and five samples of diphtheria antitoxin, each containing 1:10,000 parts Mercurophen and incubating at 37°C. for three weeks, all were found to be sterile. These were then divided into two lots, one exposed to the air and sunlight, and the other exposed to the air but protected from sunlight. At the end of a month both lots were sterile. It was

assumed from this experiment that light does not affect the germicidal power of Mercurophen.

Discussion

The germicidal power refers to the destruction of all the species of bacteria employed in the experiments. A large inoculum was intentionally used to provide for exceptionally heavy bacterial growth, one that would far surpass the usual conditions of accidental contamination. If the dilutions are not made in such a manner as to contain sufficient medium to promote growth of the bacteria, the germicidal effectiveness is given a high and false value.

The germicidal power of each substance decreased with an increasing amount of organic material present in the medium employed. There was also noted a selective action of the chemical substances, the organic mercurials being particularly effective against *Staphylococcus albus*. The tests were repeated a number of times and the germicidal and physical effects were regularly found to be as stated.

Of the chemicals studied those best suited for biologic preservatives from the standpoint of germicidal efficiency, physical effect, and the appearance of the product were Mercurophen, Metaphen, Trikresol, and phenol, in the order given. The remainder of the paper is concerned with a further study of these substances.

II. TOXICITY TESTS

This phase of the work was undertaken chiefly to determine the toxicity of Metaphen and Mercurophen, but included in part the toxicity of phenol and Trikresol. The toxicity of phenol and Trikresol has been determined by Leake and Corbett (1917) and a number of other workers, with comparable results.

Leake in 1917 used mice as experimental animals. Assuming the minimum lethal dose to be the amount necessary to kill 80 per cent of the mice at a given dose, Leake found that phenol has an M.L.D. of 0.00037 gram per gram of mouse. Trikresol has the same M.L.D. as found for phenol. The toxicity of Trikresol and phenol was not lessened when diluted with normal horse serum.

Methods

The experimental animals employed in this part of the problem were rabbits and albino rats. The injections were made intravenously and intramuscularly.

The phenol was weighed and diluted to the required concentration with double distilled water. Trikresol, Mercurophen and Metaphen in the liquid state were diluted to the desired concentration with distilled water. The quantity of each germicide to be injected into each animal was calculated by multiplying the weight of the rat or rabbit by the dose in grams of the germicide and dividing the total by the grams of germicide per cubic centimeter of solution. In the rat, intravenous injections were made in the saphenous vein, while intramuscular injections were made in the fleshy portion of the hind leg. With rabbits, intravenous injections were made in the marginal ear veins. The rats were between 100 and 150 grams in weight, the weight being taken before a heavy feeding. Rabbits were not less than 1.5 kgm. in weight and were apparently in excellent physical condition. The amounts injected were measured in 2 cc. Luer syringes, graduated in 0.1 cc., and also a 1 cc. syringe graduated in 0.01 cc. The quantity to be injected governed the choice of the syringe to be used.

The maximum tolerated dose (M.T.D.) was employed to demonstrate the tolerance of the experimental animals for the germicides studied. This degree of toxicity was determined rather than the minimum lethal dose as it was thought that it would represent more satisfactorily the margin of safety that would be essential for the safe employment of each respective germicide in the capacity of a biologic preservative. In these experiments the M.T.D. was considered to be the maximum amount of germicide necessary to kill 60 per cent of the test animals within a fourteen-day period. Animals living fourteen days after the time of injection were discharged as survivors.

Dilutions of germicides

Toxicity tests on phenol and Trikresol were carried only far enough to compare their reactions with those produced by Metaphen and Mercurophen.

The M.T.D. for both phenol and Trikresol in rats was 150 mgm. per kilogram intravenously, and 250 mgm. intramuscularly. Further tests for the M.T.D. were discontinued because of the extreme tremors manifested with such low dosages as 30 mgm. intravenously and 70 mgm. intramuscularly. Many times these symptoms appeared before the injection was completed.

Toxicity of Metaphen and Mercurophen

In determining the M.T.D. of these substances a number of factors were considered. Of first importance was the strain of rats used in the tests. Albino rats of the strain usually employed in the standardization of arsphenamine at the National Institute of Health were used exclusively. Parallel experiments carried out on rats of another strain showed a lower tolerance for the germicides than is here reported, the difference occasionally being as much as 40 per cent on identical dosages. Furthermore, during periods of intense heat, animal tolerance for the mercury preparations when injected intramuscularly or intravenously was greatly lessened. Both substances injected intramuscularly in doses of 12 mgm. per kilogram or more produced some irritation, which was manifested for several minutes. It was stated by Kolmer (1926) that solubility in the tissue, percentage of mercury, and rate of dissociation of mercury influence toxicity and physiological activity. The times of death in the toxicity experiments on animals poisoned by the mercury compounds fell into the groups as outlined by McNider (1924), depending upon the reaction and mode of administration of the mercurial substance. In the first group were animals which died usually within two days. The second group would generally survive this period but die within seven days, and a final group survived both periods, but died within fourteen days—manifesting a delayed poisoning.

Discussion

It is evident that the M.T.D. for the mercury compounds is not a constant value. As shown by the effect of dilution and the tolerance of individual animals, there is a broad range of susceptibility. In some preliminary experiments an individual animal

TABLE 1

Toxicity charts of Metaphen and Mercurophen

PREPARATION	TEST ANIMAL	STRENGTH OF SOLUTION	NUMBER OF ANIMALS ON EACH DOSE	DOSAGE	ROUTE OF INJECTION	PER CENT SURVIVAL	M.T.D.
		per cent		mgm. per kgm.			mgm. per kgm.
Metaphen	Albino rat	1 0	5	16.6	Intramuscular	100	
		1.0	5	20.0	Intramuscular	100	
		1.0	5	24.0	Intramuscular	60	24 0
		1 0	5	28 0	Intramuscular	0	
		2 0	4	14 0	Intramuscular	100	
		2 0	4	16.6	Intramuscular	100	
		2 0	4	20 0	Intramuscular	75	20.0
		2 0	4	24 0	Intramuscular	0	
		0.5	4	2 6	Intravenous	100	2.6
		0 5	4	4 0	Intravenous	50	
		0 5	4	6 0	Intravenous	0	
		0.1	5	4.0	Intravenous	100	
		0.1	5	6 0	Intravenous	60	6.0
		0 1	5	8.0	Intravenous	0	
	Rabbit	2 0	4	6 0	Intramuscular	100	
		2 0	4	8 0	Intramuscular	100	
		2 0	4	10 0	Intramuscular	75	12 0
		2.0	4	12 0	Intramuscular	60	
		2 0	4	14 0	Intramuscular	25	
		2.0	4	16 0	Intramuscular	25	
		2 0	4	3 0	Intravenous	100	
		2 0	5	4 0	Intravenous	80	4.0
		2.0	4	5 0	Intravenous	25	
		2.0	4	6 0	Intravenous	0	
Mercurophen	Albino rat	1 0	5	16 6	Intramuscular	100	
		1.0	5	20 0	Intramuscular	80	
		1.0	5	24 0	Intramuscular	60	24.0
		1.0	5	28.0	Intramuscular	0	
		2.0	4	14 0	Intramuscular	100	
		2.0	4	16 6	Intramuscular	80	16 6
		2 0	4	20 0	Intramuscular	20	
		2.0	4	24 0	Intramuscular	0	
		0.5	5	4.0	Intravenous	80	4.0
		0 5	5	6.0	Intravenous	0	
		0 5	5	8.0	Intravenous	0	
		0 1	5	8 0	Intravenous	100	8.0
		0.1	5	10 0	Intravenous	20	
		0 1	5	12 0	Intravenous	0	

TABLE 1—*Concluded*

PREPARATION	TEST ANIMAL	STRENGTH OF SOLUTION	NUMBER OF ANIMALS ON EACH DOSE	DOSAGE	ROUTE OF INJECTION	PER CENT SURVIVAL	M.T D.
		per cent		mgm. per kgm.			mgm. per kgm.
Mercurophen —*Concluded*	Rabbit	2.0	4	6 0	Intramuscular	100	
		2.0	5	8.0	Intramuscular	80	
		2.0	5	10.0	Intramuscular	80	
		2.0	4	12.0	Intramuscular	75	12 0
		2.0	4	14.0	Intramuscular	50	
		2.0	2	16.0	Intramuscular	0	
		2.0	4	3.0	Intravenous	100	
		2.0	5	4.0	Intravenous	80	4.0
		2.0	4	5.0	Intravenous	25	
		2 0	4	6.0	Intravenous	9	

would occasionally tolerate a dose as great as 40 mgm. of Metaphen or Mercurophen per kilogram. The higher concentrations may produce thrombosis and cause sudden death. It will be noted in the intravenous experiments in rats that when 0.5 per cent solutions were employed, death occurred generally within twenty-four hours, giving a dosage toleration of 50 per cent less than that of the 0.1 per cent solution. From these data it may be inferred that dilution influences the susceptibility of the animals. This is particularly true when the injection is intravenous.

The M.T.D. of Metaphen and Mercurophen in rabbits was found to be 4 mgm. per kilogram intravenously and 12 mgm. per kilogram intramuscularly. The albino rats showed a tolerance to the mercury compounds of 0.1 per cent solution intravenously and 1.0 per cent solution intramuscularly as follows: Metaphen, intravenously 6 mgm. per kilogram; intramuscularly 24 mgm. per kilogram; Mercurophen, 8 mgm. per kilogram intravenously, and 24 mgm. per kilogram intramuscularly. Rats showed 100 per cent greater tolerance to Mercurophen intravenously for the 0.1 per cent solution than for the 0.5 per cent solution. Rabbits had a lower tolerance both for intravenous and intramuscular injections than that exhibited by rats. Phenol and Trikresol are approximately 25 times less toxic intravenously and 10 times less

TABLE 2

Toxicity charts of phenol and Trikresol

PREPARATION	TEST ANIMAL	STRENGTH OF SOLUTION	NUMBER OF ANIMALS ON EACH DOSE	DOSAGE	ROUTE OF INJECTION	PER CENT SURVIVAL	M.T.D.
		per cent		mgm. per kgm.			mgm. per kgm.
Phenol	Albino rat	2.0	2	10.0	Intravenous	100	
		2.0	2	20.0	Intravenous	100	
		2.0	2	30.0	Intravenous	100	
		2 0	2	40.0	Intravenous	100	
		2 0	2	70.0	Intravenous	100	
		2.0	3	100.0	Intravenous	100	
		2.0	3	120.0	Intravenous	100	
		2.0	3	130 0	Intravenous	66	150
		2 0	3	150.0	Intravenous	66	
		2.0	3	170.0	Intravenous	0	
		2.0	3	200.0	Intramuscular	100	
		2.0	3	220.0	Intramuscular	100	
		2.0	3	240.0	Intramuscular	100	
		2.0	3	250.0	Intramuscular	100	250
		2.0	3	260.0	Intramuscular	33	
		2.0	3	270.0	Intramuscular	0	
Trikresol	Albino rat	2.0	2	10 0	Intravenous	100	
		2.0	2	20.0	Intravenous	100	
		2.0	2	30.0	Intravenous	100	
		2.0	2	40.0	Intravenous	100	
		2.0	2	70.0	Intravenous	100	
		2.0	3	100.0	Intravenous	100	
		2.0	3	120.0	Intravenous	100	
		2.0	3	130.0	Intravenous	100	
		2.0	3	150.0	Intravenous	66	150
		2.0	3	170.0	Intravenous	0	
		2.0	3	200.0	Intramuscular	100	
		2.0	3	220.0	Intramuscular	100	
		2.0	3	240.0	Intramuscular	66	
		2.0	3	250.0	Intramuscular	66	250
		2.0	3	260.0	Intramuscular	0	
		2.0	3	270.0	Intramuscular	0	

toxic intramuscularly than Metaphen and Mercurophen. However, the M.T.D. is misleading in the case of phenol and Trikresol because of the extreme tremors manifested and the "burning" of

the tissue of the animals in amounts far below the maximum tolerated dosage.

If the toxicity of Metaphen and Mercurophen is placed upon a comparative basis, there is little difference between them. Mercurophen appears to be somewhat less toxic than Metaphen when used intravenously in rats but both have the same M.T.D. in rabbits.

III. INTRASPINAL TESTS.   TO TEST THE ACTION OF METAPHEN AND MERCUROPHEN AS COMPARED WITH THE ACTION OF PHENOL AND TRIKRESOL WHEN INJECTED INTRASPINALLY

In 1913, Kramer (1913) observed a number of cases of sudden death after the treatment of cerebro-spinal fever with intraspinal injections of specific antiserum, and advanced the hypothesis that the preservative, Trikresol, was the cause of the symptoms.   That the toxic action of the serum introduced into the subarachnoid space of dogs and cats was due to this preservative was later proven by Hale (1913).   In 1914, Voegtlin (1914) confirmed the results of Kramer and of Hale and found that Trikresol and phenol were equally toxic when injected in like quantities, and stated, "As a general rule, the effect on blood pressure increases in proportion to the rate of injection and the concentration of the phenol preservative."

The fact that phenol and Trikresol possess such toxic properties when injected intraspinally made it desirable to know the effects produced by Metaphen and Mercurophen upon the central nervous system.

Procedure

The experiments were carried out on dogs.   The anesthetic employed was ether and the degree of anesthesia was kept uniform by performing tracheotomy and introducing the vapor from a bottle directly into the trachea.   Blood pressure was taken from the carotid artery, and tracings were obtained by using a mercury manometer.   Respiratory rates were recorded graphically by inserting a canula into the pleural cavity just above the diaphragm and recordings made on a kymograph connected with a tambour.

Methods of injection

Two methods of injection were employed; namely, by pressure and by gravity. Several vertebrae in the lumbar region were exposed and injections made by introducing a needle directly into the spinal canal. Spinal fluid was permitted to escape. The needle was then connected with a Luer type of syringe or graduated burette depending upon the method used.

Antimeningococcus serum was preserved with Mercurophen and Metaphen in 1:2,500 and with phenol and Trikresol in 1:200 concentrations. These dilutions permitted a margin of more than double the quantity of Metaphen and Mercurophen needed for preservation purposes. Sera preserved with each of the above substances were injected alternately, using both the pressure and gravity methods of administration.

Protocol

Only one protocol of several experiments is given since the others are similar to this one. The symptoms manifested after the administration of phenol intraspinally are the same as those produced by Trikresol, so a protocol showing in detail the effect of the latter substance is not included.

Dog 2, 8.2 kgm.   Anesthetic, ether.

10:32 a.m., withdrew 1.5 cc. spinal fluid—clear.

10:41 to 10:50 a.m., 10 cc. serum preserved with Metaphen 1:2,500 injected intraspinally by gravity method.

10:59 a.m., pressure released and serum permitted to flow out. Blood pressure and respiration normal.

11:10 to 11:12 a.m., 4.1 cc. serum containing 0.5 per cent phenol administered by gravity method. A drop in blood pressure followed, with shallow and uneven respiration. Immediately before releasing injection pressure, coarse tremors were manifested. After releasing the serum from the spinal canal, there was an immediate rise in blood pressure to normal and respiration became constant.

11:27 to 11:30 a.m., 4 cc. of one part Mercurophen, in 2,500 parts serum administered by gravity method.

11:30 to 11:33 a.m., 1.5 cc. additional serum as above.

11:34 a.m., injection pressure released. Blood pressure normal,

respiration normal.   No change in respiration and blood pressure after the release of the preserved serums.

11:45 to 11:52 a.m., 4 cc. serum containing Mercurophen (1:2,500) administered by gravity method.   Blood pressure and respiration normal.

12:01 to 12:06 p.m., 4.2 cc. serum containing phenol (0.5 per cent) administered by gravity method.   A slight rise in blood pressure was followed by a gradual fall.   When injection pressure was released, blood pressure became normal.

12:10 to 12:11 p.m., 2.5 cc. serum containing phenol (0.5 per cent); rapid decrease in blood pressure, respiration normal.

12:19 to 12:23 p.m., 9 cc. 1 part Metaphen in 2,500 parts serum injected under pressure.   Blood pressure and respiration normal.

12:27 to 12:29 p.m., 2.9 cc. serum containing phenol (0.5 per cent) injected by gravity.   Gradual decrease in blood pressure returning to normal when injection pressure was released.

12:45 to 12:47 p.m., 7 cc. serum containing phenol (0.5 per cent) injected under pressure.   Marked decrease in blood pressure, respiration became shallow and finally stopped; injection pressure was released after which the respiration and blood pressure became normal.

12:54 to 12:58 p.m., normal salt solution containing phenol (0.5 per cent) administered by pressure.   Marked decrease in blood pressure, tremors were shown, respiration became shallow and finally ceased.

Discussion

Phenol and Trikresol.   The results obtained with a serum containing phenol and Trikresol (1:200 parts), were in general analogous to those of Voegtlin (1914).   In all cases there was an immediate and marked drop in blood pressure with a slowing and final arrest of respiration if the pressure was not released.   Serum injected under pressure gave a more precipitous drop than when injected by the gravity method.   The amount of phenolized serum necessary to cause these effects was small and within several minutes' time.   Respiratory and blood pressure changes are shown in figures 1 and 2.

The severe symptoms manifested in the vasomotor and respiratory centers with phenol and Trikresol makes the use of these substances as preservatives in biologic products to be administered via the spinal canal a hazardous one.

Metaphen and Mercurophen. A review of the protocols and the respiratory and blood curves after the injection of these two diluted mercurial compounds showed an absence of the severe symptoms manifested in the phenol experiments. Injections of
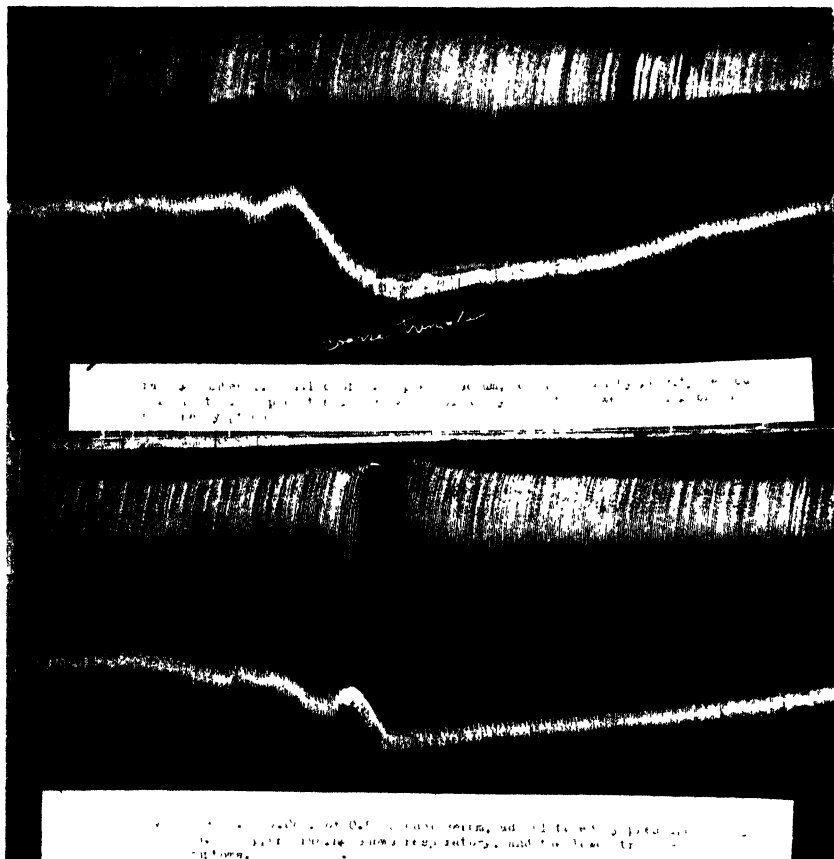
FIGS. 1 AND 2

large amounts of serum preserved with Metaphen and Mercurophen had no noticeable effect upon the blood pressure or respiratory movements as shown in figures 3 and 4. The pressure method of injection produced a slight increase in the blood pressure but the symptoms shown would indicate that these sub-

stances were not dangerous. It may, therefore, be assumed that neither Metaphen nor Mercurophen may be expected to have any harmful effects upon the vasomotor or respiratory centers in the amounts present in the usual doses of therapeutic sera.

Tracing Number 4. 4cc. of 1-2500 parts mercurophen and serum, which entered by gravity method in 7 minutes. Upper tracing respiration; lower tracing blood pressure.

Tracing Number 5. 10cc. of 1-2500 parts metaphen and serum, which entered by gravity method in 12 minutes. Upper tracing respiration; lower tracing blood pressure.
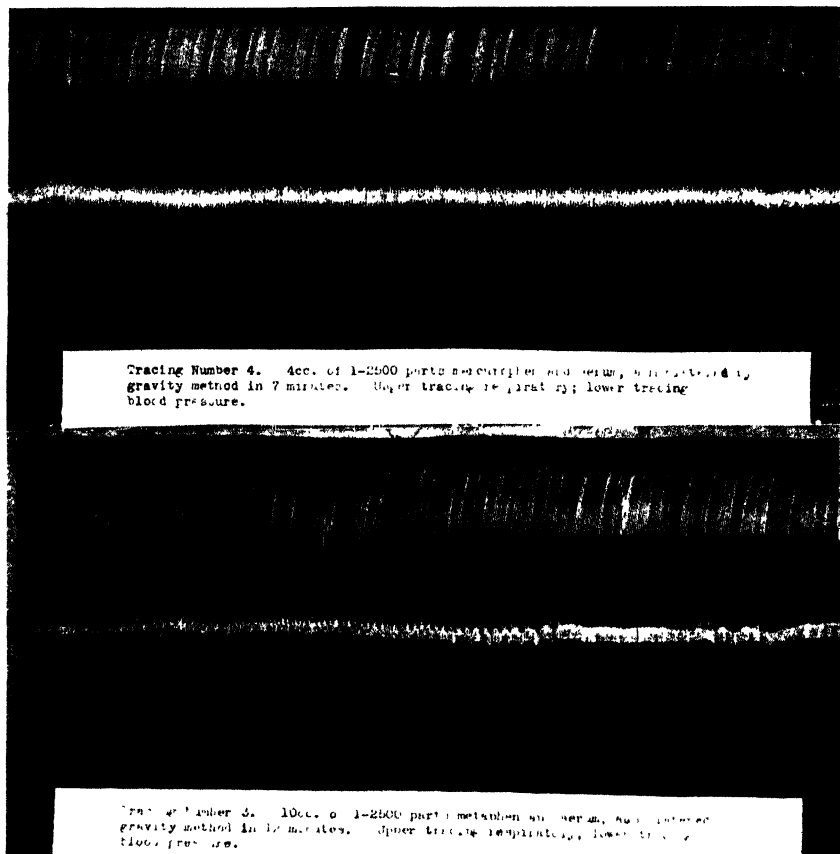
Figs. 2 and 3

IV. POTENCY TESTS

Sera without preservative were collected from various manufacturing laboratories for this phase of the study. Tetanus toxin and antitoxin, and diphtheria toxin and antitoxin were used to determine the effect of the germicidal substances on their toxicity

or potency. To the above toxins and antitoxins were added the quantities of each preservative which had been found to be germicidal. The substances used and quantities added were:

Phenol, 0.5 per cent in toxins and antitoxins
Trikresol, 0.4 per cent in toxins and antitoxins
Metaphen, antitoxins 1:5,000, toxins 1:10,000
Mercurophen, antitoxins 1:8,000, toxins 1:12,000

These mixtures were then placed in a refrigerator at 4.5°C. together with the controls of unpreserved serum. A preliminary test was made on these toxins and antitoxins to determine their exact toxicity or unitage. They were then placed in an incubator at 37°C. to hasten any action of the preservatives. At intervals, potency tests were made on preserved and unpreserved sera to note any decrease in potency.

Potency tests. The methods employed in testing the potency of the biologic products studied were essentially the same as those used at the National Institute of Health in testing the potency of standard commercial diphtheria and tetanus toxins and antitoxins.[3]

The strength of the toxins was measured in terms of M.L.D.'s per cubic centimeter, being the minimal amount of toxin necessary, when injected subcutaneously in the test animals of a given weight, to produce acute toxic death in approximately ninety-six hours. The potency of antitoxin was measured in units, a unit being the minimal amount of antitoxin which when added to 1 L+ dose of toxin and injected subcutaneously into a test animal of given weight would protect the test animal for approximately ninety-six hours.

Discussion

As a result of these experments the following conclusions seem to be warranted:

Diphtheria toxin, when preserved with phenol, Trikresol, Metaphen or Mercurophen, showed no decrease in strength when

[3] Tests of standard and commercial diphtheria and tetanus antitoxin. Published by the United States Hygienic Laboratory (Laboratory memorandum).

compared to that of unpreserved toxin after sixteen and twenty-eight days' incubation at 37°C.

Diphtheria antitoxin similarly preserved showed no loss of potency when compared to the potency of the unpreserved serum when incubated at 37°C. for sixteen, twenty-two and thirty-nine days.

Incubation at 37°C. for fourteen days caused a complete loss of toxicity in the control and preserved tetanus toxin while tetanus antitoxin preserved as above retained its potency for the periods studied, i.e., seven and fourteen days.

An analysis of the potency tests showed similar results with no definite evidence of loss of toxicity or potency due to the action of any of the chemical substances used as preservatives. Tetanus toxin, however, is unstable at 37°C. and the unpreserved as well as the preserved product showed a complete loss of toxicity.

V. HISTOPATHOLOGIC STUDY OF THE EFFECTS OF METAPHEN AND MERCUROPHEN UPON TISSUES

The ability of certain mercury salts to produce nephritis has been known for years.

In 1860 Pavy induced an experimental nephritis with mercury. He considered the Malpighian bodies to be the seat of injury and also noted necrosis of the tubular epithelium. Later, Aschoff (1912) reported in detail the renal pathology produced by mercuric chloride, which consisted in swelling, hyaline vacuolation, and necrosis of the tubular epithelium.

The present study was undertaken to learn if the mercury compounds, Metaphen and Mercurophen, in doses representing 25 per cent of the M.T.D., produced any tissue changes.

Procedure. For this study albino rats were used as the experimental animals. One hundred and fifty per cent and 25 per cent of the maximum tolerated doses of Metaphen, Mercurophen, and mercuric chloride were administered intramuscularly. These doses were chosen in order that the lesions produced by a toxic dose and sub-tolerated dose might be learned. Mercuric chloride was also employed so that a comparative study of the lesions produced by Metaphen and Mercurophen might be made.

The animals were killed at varying lengths of time in an attempt to observe the various stages of the lesions produced. Six rats were used on each mercurial substance in the 150 per cent dosage and six rats on the 25 per cent maximum tolerated dosages. With the latter dosage the animals were killed on the fifth, seventh, twelfth, fifteenth, seventeenth, and twenty-first days. Care was taken to place the tissues in fixing fluids immediately after the death of the animal.

Discussion

The administration of 150 per cent of the maximum tolerated doses of Metaphen, Mercurophen, and mercuric chloride produced tissue injuries which were essentially identical in character and in degree. Lesions of the heart muscle, spleen, and liver were not constant, and when demonstrated at all, were of minor importance. The kidney showed the most characteristic changes; namely, a toxic nephrosis.

Mercuric chloride produced necrosis of the muscular tissue at the site of injection in two animals. This condition was not found in any of the rats injected with Metaphen or Mercurophen.

It was not possible to differentiate the lesions produced by the three mercurials studied. The degree of tissue injury produced depended apparently on the amount of mercury administered and the length of survival. One-fourth of the M.T.D. produced no evident histologic changes. The quantity of germicide which would be present in the maximum dose of serum to be administered is materially less than one-fourth of its maximum tolerated dose. Therefore, because of the extremely small amount of the two mercury compounds, Metaphen and Mercurophen, needed to preserve biologic products, these substances may be used for this purpose without fear of causing any symptoms or pathologic changes in persons receiving the maximum therapeutic doses of these serums.

SUMMARY

Yatren and Hexylresorcinol (1:1,000) have insufficient germicidal power to permit their use for the preservation of biologic products.

Colloidal chloro-cresol and colloidal chloro-thymol, either in 25 or 50 per cent strengths, are unsuitable as biologic preservatives, because of their destructive action in precipitating protein.

In serum, Mercurophen has an approximate germicidal potency 40 times greater, and Metaphen 25 times greater, than phenol and Trikresol. Intramuscular injections in rats of 1 per cent solutions of Metaphen and Mercurophen are approximately 10 times more toxic than phenol and Trikresol.

The higher germicidal power of these two mercury compounds causes them to be relatively less toxic than phenol and Trikresol when used as biologic preservatives, and permits a greater margin of safety in their use.

Neither Metaphen nor Mercurophen at their germicidal strength precipitates proteins, while both phenol and Trikesol have this action.

The use of phenol and Trikresol in germicidal concentrations in the spinal canal is dangerous because of action upon the respiratory and vasomotor centers. Serums preserved with Metaphen and Mercurophen, on the other hand, when injected into the spinal canal had no effect upon the blood pressure or respiratory movements.

It was demonstrated that the type of tissue injury produced by Metaphen and Mercurophen is entirely dependent upon the quantity of mercury administered. Quantities representing 25 per cent of the maximal tolerated dose produce no histologic changes.

As far as could be determined neither phenol, Trikresol, Metaphen, nor Mercurophen decreases the potency of toxins and antitoxins.

Of the biologic preservatives studied, the mercury compound, Mercurophen appears to be the most satisfactory, with Metaphen as second choice, and both superior to phenol and Trikresol.

REFERENCES

Aschoff, L. 1912 Zur Morphologie der Nierensekretion unter physiologischen und pathologischen Bedingungen. Verhändl. d. Deutschen Pathologische Gesellschaft, 199.

Hale, Worth 1913 The cause of death from subdural injections. Hygienic Laboratory Bulletin, No. 91, p. 5.

Kolmer, John A. 1926 Principles and practice of chemotherapy with special reference to the specific and general treatment of syphilis. W. B. Saunders Company, Philadelphia, 739.

Kramer, S. P. 1913 A possible source of danger in the use of antimeningitis serum. Jour. Amer. Med. Assoc., 60, 1348.

Laubenheimer, Kurt 1928 Ueber Chlorothymol als Desinfektionsmittel. Aus dem Staatsinstitut für experimentelle Therapie in Frankfurt, a.M.

Leake, James P., and Corbett, Hugh B. 1917 Toxicity of certain preservatives used in serums, viruses, and vaccines. Hygienic Laboratory Bulletin, No. 110, p. 35.

MacNider, W. deB. 1924 A review of acute experimental nephritis. Physiol. Rev., 4, 595.

Pavy, F. W. 1860 Poisoning by white precipitate. Guys Hospital Reports, London, p. 504.

Raiziss, G. W., and Severac, M. K. 1923 A new organic mercury compound with powerful germicidal properties. Jour. Lab. and Clin. Med., 9, 71.

Voegtlin, Carl 1914 The pharmacological action of some serum preservatives. Hygienic Laboratory Bulletin, No 96, p. 87.

PATHOGENICITY AND CLASSIFICATION OF MICROÖRGANISMS[1]

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Two factors determine the character of diseases caused by microörganisms: the reaction of the host and the qualities of the pathogenic organisms. The reaction of the host depends on the species, as different species react differently to the same microorganism, e.g., reaction of man and animals to typhoid bacteria. Different organs of the same individual respond in a different way as seen in the reaction of skin and heart to streptococci. The reaction of the same organ may be more or less similar to different classes of microörganisms, e.g., the reaction of the large intestine to *Eberthella dysenteriae* and to *Amoeba dysenteriae*.

The botanical characteristics of pathogenic microörganisms, however, determine the general character of the response of the diseased organism (see Pribram, 1929).

There is in the first place one group of microörganisms, which cause a marked local and general leucocytosis of the host, staphylococci, streptococci, *Neisseria gonorrhoeae*, *Neisseria catarrhalis*, *Neisseria intracellularis*, *Streptococcus lanceolatus*, further, Escherichias and Salmonellas, *Eberthella dysenteriae*, *Vibrio cholerae*, *Pseudomonas pyocyanea* and *alcaligenes*. The common botanical qualities of these microörganisms are their low differentiation and easy adaptation to the animal organism. They are not very resistant, they are with few exceptions—staphylococci and streptococci—Gram-negative and multiply readily in the host. The local response to these organisms does not differ qualitatively from the response to the presence of any foreign protein. It is

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[1] Read before the American Association of Immunologists in Cleveland, Ohio, on April 1, 1931.

an acute inflammation, aggregation of leucocytes, formation of abscesses. The general response of the host is a leucocytosis with a prevalence of granulocytes. The metabolic products of all these microörganisms act upon the myelogenous system. This also applies to those microörganisms, to which the host responds with a leucopenia, such as *Eberthella typhi*, *Brucella melitensis*, *Hemophilus influenzae*.

There is a second group of microörganisms with a more marked action upon the lymphatic and the reticulo-endothelial system besides their action on the myelogenous system. We find a general reaction of the lymphatic system in the disease caused by *Hemophilus pertussis*. We further find very pronounced local reactions in the diseases caused by the Pasteurella group. The main botanical characteristics of this group is a more resistant body (bipolar staining). Their biological action on the lymphatic system causes a hyperplasia of the lymph glands. They have also a strong lytic action on the wall of the blood vessels. They cause hemorrhages. The best example for human diseases of this group is the plague, caused by *Pasteurella pestis*. The acute form, Black Death, is characterized by leucocytosis with prevalence of granulocytes and hemorrhages in the lungs or in the lymph glands (bubos). During the chronic stage the monocytes prevail, and these come from the reticulo-endothelial system. The botanical position of Pasteurella leads directly to the genus Malleomyces, which I classified on account of its branching with the family Mycobacteriaceae. The difference between the bubo caused by *Pasteurella pestis* and the nodules caused by *Malleomyces mallei* is striking. The bubo in the acute stage of plague shows lymphatic hyperplasia, edema, leucocytosis and hemorrhagic areas, later necrosis and monocytes (macrophages). The nodules in glanders are formed by epithelioid cells; they are later invaded by leucocytes, which gradually liquefy the center. The final histological picture shows a purulent liquefied center surrounded by epithelioid and spindle-shaped cells. In chronic glanders the leucocytes are absent and epithelioid cells prevail. The nodules in the spleen contain epithelioid cells and even giant cells may be present of the Langhans type. The botanical position of *Mal-*

leomyces mallei approaches the position of *Mycobacterium tuberculosis* and so does its pathogenic character. The study of another species of Malleomyces, which is usually classified with Pasteurella, *Bacterium pseudotuberculosis-rodentium*, revealed that its cells easily ramify (Skschivau, 1900; Rosenfeld, 1901). I classified it therefore with the genus Malleomyces. This microörganism is Gram-negative and not acid-fast. It causes in rodents a disease, which resembles miliary tuberculosis. The nodules consist of epithelioid and lymphoid cells with a purulent or caseated center. They do not contain giant cells of the Langhans type. They never calcify. We deal here with another species of the genus Malleomyces, which in its pathogenic character comes still closer to the species *Mycobacterium tuberculosis* than does *Malleomyces mallei*.

Corynebacterium is the Gram-positive genus of the order Mycobacteriales which corresponds to the Gram-negative genus Malleomyces. If we do not take account of the toxin production of the species *Corynebacterium diphtheriae* we find the following local destruction of the tissue invaded by this microörganism: Thrombofibrosis of the vessels with a secondary formation of pseudomembranes; also nodules with epithelioid cells and necrosis present in the spleen and in the lymphoid follicles of the intestinal tract.

Fusiformis vincenti is another microörganism which attacks the lymphoid tissue and produces membranes similar to those of *Corynebacterium diphtheriae*, sometimes also nodules resembling those of tuberculosis. The general reaction of the host to Fusiformis is a pronounced lymphocytosis. The botanical position of the genus Fusiformis of the family Bacteroidaceae corresponds to the position of the genus Corynebacterium in the family Mycobacteriaceae. The fusiformis organism is unbranched, Gram-positive, anaerobic; Corynebacterium is branched, aerobic and also Gram-positive.

Mycobacterium tuberculosis combines those pathogenic characteristics which are directed against the lymphatic and reticuloendothelial system, with almost no action upon the myelogenous system. It causes a general lymphocytosis and a strong local

reaction of the reticulo-endothelial cells resulting in the formation of the typical tubercle, starting with a thrombofibrosis of the vessels: There is a necrosis (caseation) in the center, with epithelioid cells and giant cells, surrounded by round cells. *Mycobacterium leprae*, closely related to *Mycobacterium tuberculosis* has a similar action, with a more pronounced tendency to produce granulation tissue, even granuloma. These are products of spindle-shaped epithelioid cells resembling almost the cells of sarcoma. The botanical position of the genus Mycobacterium is that of a transition form between the class Schizomycetes and the class Eumycetes (fungi).

Mold infections are of a twofold type. Those which invade the deeper layer of the tissue produce granulation tissue (granulomas), e.g., Actinomyces, Sporotrichon. In Actinomyces infection there is a tendency to a lipoid metamorphosis of the cells. There is in all mold infections an intense irritation of the reticulo-endothelial system with no, or only a slight, reaction of the lymphatic system. The lymph glands are rarely involved. Those molds, which invade the superficial layer of the skin or mucous membranes destroy the epithelial layer and form pseudomembranes (Candida, Cryptococcus, etc., in thrush). There is no effect on the lymphatic system and only a slight irritation of the reticulo-endothelial system.

Returning to the class Schizomycetes we have further the order Bacillales, characterized by the formation of endospores. There is one aerobic representative in this order, *Bacillus anthracis* and several anaerobic species of the genus Clostridium, *Clostridium welchii, Clostridium oedematis-maligni, Clostridium thermophilum, Clostridium chauvoei (Cl. anthracis-symptomatici)*. We find a common pathogenic action in all the diseases caused by these microörganisms. They all produce edema and necrosis of the tissue which they attack. According to the production of different ferments there are differences in the reaction, such as gas production, gangrene, etc. Those anaerobic spore formers, such as *Clostridium tetani* and *Clostridium botulinum,* which do not multiply readily in the host do not produce local reactions, but act almost exclusively by their toxins on the nervous system.

The order Spirochetales, which are the transition forms between Protozoa and Schizomycetes, have according to this position a pathogenic action similar to the action of certain species of the class Protozoa. Some of them are exclusively blood parasites, e.g., *Spirochaeta recurrentis*. They have an action similar to that of *Plasmodium malariae* or *Trypanosoma naganae*. Another group invades the tissue, e.g., *Treponema pallidum*. They produce diseases in man which resemble diseases of animals caused by *Trypanosoma equiperdum* and *Trypanosoma equinum*. Their primary attack causes an intense reaction of the lymphatic system. The local reaction as well as the general reaction are limited to the lymph and blood vessels, which relax and become surrounded by round cells and plasma cells of lymphatic origin. This reaction causes the local induration (sclerosis) as well as the syphilitic bubos, the lymphocytosis and the different forms of exanthem. There is also, always, in the later stages of the disease a prevalence of the lymphatic reaction over the reaction of the reticular cells, a prevalence of fibroblasts over the epithelioid type; fibrous aortitis, periarteritis nodosa and gummosa, the formation of gummas exemplifying of the tendency to form fibrotic tissue. Tabes and paralytic dementia are secondary lesions caused mainly by the fibrotic changes of the blood vessels.

SUMMARY

There is a certain relation between pathogenicity of microörganisms and their botanical position.

Microörganisms of a relatively low degree of differentiation, such as Micrococcaceae, pathogenic Pseudomonadaceae, pathogenic Aerobacteriaceae of the class Schizomycetes and the pathogenic Amoebas of the class Protozoa act merely as foreign proteins producing a strong reaction of the myelogenous system, local pus-formation, general leucocytosis.

Microörganisms of a higher degree of differentiation attack besides the myelogenous system also the reticular and endothelial and lymphatic systems. Pasteurellaceae have a marked action upon the vessels, causing hemorrhages, and upon the reticulo-endothelial tissue in the chronic stage of the diseases (monocyto-

sis). The pathogenic Mycobacteriaceae, Corynebacterium, Malleomyces, Mycobacterium, have an increasing action upon the reticulo-endothelial system, the more they approach in their botanical characteristics Mycobacterium and the fungi. The action of Mycobacterium is limited to the reticulo-endothelial and to the lymphatic system. There is a prevalence of the response of the endothelial system and a thrombofibrosis of the vessels.

The molds, Actinomyces, Sporotrichon, have a still more selective action on the endothelial system, causing granulomas. Some molds, Candida for instance, simply may grow as parasites destroying the superficial cells and producing pseudomembranes.

The spore-forming order Bacillales with the genera Bacillus and Clostridium cause edema of the subcutaneous and muscular tissue, if they proliferate abundantly. The majority of the genera of this order do not grow or grow but slowly in animals. They are soil bacteria.

Spirochaetales and the more highly differentiated Protozoa, such as *Plasmodium malariae* and Trypanosomas are either blood parasites or attack almost exclusively the lymphatic and vascular system. They produce a hyperplasia of the lymph glands, a lymphocytosis, formation of fibroblasts and finally fibrosis of the walls of the vessels.

REFERENCES

PRIBRAM, E. 1929. A contribution to the classification of microörganisms. Jour. Bacteriol., **18**, 361.

ROSENFELD. 1901. Centralbl. f. Bacteriol., **30**, 642.

SKSCHIVAU. 1900. Centralbl. f. Bacteriol., **28**, 289.

THE RELATIVE PERSISTENCE OF BACT. COLI AND BACT. AEROGENES IN NATURE

I. IN DECAYED STUMPS

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A growing question for practical consideration in water control is the relative sanitary significance of *Bact. coli* and *Bact. aerogenes* as criteria of fecal pollution. It is generally agreed that *Bact. coli* is strictly of fecal origin, and its presence in water therefore is accepted as evidence of dangerous pollution. *Bact. aerogenes*, however, occupies a doubtful position as an organism of fecal origin. Some observers regard it as of the same fecal significance as *Bact. coli*, while others consider it of little, if any, value as indicating contamination from fecal sources. There appears to be growing evidence in support of the latter view (Chen and Rettger (1920); Koser (1927); Hinman (1925); Tonney and Noble (1930)). Certainly, the great predominance of *Bact. aerogenes* over *Bact. coli* at large in nature needs some explanation other than the assumption that both are of direct fecal origin, since in feces itself the reverse relationship is true. In previous studies we have found the ratio of *Bact. coli* to *Bact. aerogenes*, by the differential plate count, to be about 100 to 1 in feces of both human and animal origin, and on the other hand about 1 to 20 in soils and vegetation (Tonney and Noble (1930)).

This wide divergence in the numerical relation of the two organisms is against the assumption that they are both of direct fecal origin and hence equally significant of fecal pollution. Possible explanations of the phenomenon, however, are:

1. That *Bact. aerogenes* survives longer and in greater numbers than *Bact. coli* outside of the animal body.

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2. That *Bact. aerogenes* at large in nature multiplies to a greater extent than *Bact. coli* in the same environment.

3. That *Bact. aerogenes* is primarily of non-fecal origin and finds its way into the animal body with the food, hence appearing irregularly in the feces as a transient rather than a characteristic organism.

In an effort to throw light on this problem, the experiments here reported were made on the relative persistence of *Bact. coli* and *Bact. aerogenes* under one set of natural conditions. The procedure consisted of planting several series of decayed stumps, some with fecal material and some with washed suspensions of the two organisms, and making differential plate counts of their relative numbers at intervals until they disappeared. By this method we were able to study the relative persistence of the two organisms exposed to the weather under identical conditions, and at all seasons of the year. Observations were made of (1) their relative rate of reduction, (2) the relative time of their survival, (3) their relative rates of growth when the conditions were such as to favor it.

Beginning December 27, 1929, and at suitable intervals as the season advanced, seven series of stumps were planted with sterile distilled water suspensions of human feces and of cultures of *Bact. coli* and *Bact. aerogenes*. The cultures were isolated, respectively, from feces and Lake Michigan water.

TECHNIC

Suspensions of the feces were made by emulsifying normal and diarrheal stools in quart bottles of sterile distilled water. Similar suspensions were prepared from culture material, washed from a suitable number of agar slants on which the organisms had been grown. Prior to the planting, sufficient material from the stump was collected for test of its colon-aerogenes content. Immediately thereafter the plant was made by carefully pouring the contents of a bottle into the hollow portions and crevices of the stump. Care was taken to assure complete absorption of the liquid by enough of the stump substance to yield sufficient sampling material over a considerable period of time. After

about thirty minutes a sample of the infected stump was collected in a sterile wide-mouthed bottle.

At suitable intervals thereafter, depending upon the season of the year and the duration of the series, further samples were taken until two successive negative specimens for each organism had been obtained.   When the samples could not be examined on the same day, they were held in the refrigerator over night.   The procedure of testing was as follows:

Twenty grams of the sample were weighed into a sterile container to which were added 200 cc. of sterile distilled water.   The contents were shaken and allowed to settle for one-half to two hours, depending upon the amount of coarse material remaining in suspension.   Five 10.0 cc. portions of the supernatant liquid were planted in ferrocyanide-citrate agar in accordance with the modified technic previously reported by us (Tonney and Noble (1931)).

The differential plate counts of *Bact. coli* and *Bact. aerogenes* thus obtained were studied for trends and relationships between the two types of organisms under the natural conditions of the experiment.

RESULTS

Since the primary purpose of the study was to show the relative persistence of *Bact. coli* and *Bact. aerogenes* exposed under identical conditions in decaying wood, no attempt was made to evaluate the probable influencing factors.   Among these factors are: (1) temperature, (2) rainfall, (3) the kind of wood, its hardness, moisture and chemical content, (4) the presence of molds and fungi, (5) the presence of other bacteria, (6) subsequent secondary infection by birds and animals, and (7) the influence of the distilled water used for suspending the samples.   It was merely assured by the conditions of the experiment that these factors occurring under natural conditions were equally operative on both organisms.

For convenience, the data are separated into two groups, those obtained from feces plantings and those from the pure cultures. The feces group is subdivided into three series while the culture group is subdivided into four series.

The feces group

In series I of the feces group, five stumps were planted with fresh human feces and two with fresh bovine feces[1] on December 27, 1929. The weather at the time was very cold and a blizzard was in progress. There was a sharp decline in the number of *Bact. coli* from the initial average of 532,000 per gram to 0 per gram on the 18th day, with small numbers recurring up to the 61st day. Unfortunately no tests were made of this group after the initial planting, until the 18th day. The initial absence of *Bact. aerogenes* in the quantities tested is noted. This organism, however, appeared on the 18th and 21st days.

Two stumps constituted series II. They were planted with feces on April 4, in the spring, and the organisms persisted through the warm season until the 153rd day (September 4), when the last recovery was made. The initial *Bact. coli* content was 11,800 per gram, with *Bact. aerogenes* absent in the quantity tested. Recovery of the latter organism, however, occurred more often than in series I and as late as the 153rd day. A difference between series I and series II is the relatively greater number of the *Bact. aerogenes*. This may be due, in part, to the warm weather.

In series III, representing ten stumps, planted with feces, there was a uniformity of ratio between the two types of organisms, with *Bact. coli* predominating, as at the outset. The magnitude of the numbers was somewhat greater in this series, which extended over the warmer season from June 7 to November 16, a total of 172 days. The larger numbers surviving may be the effect of the season, or may be due to a larger initial content or both.

In figure 1, trend curves of these data, indicate a decline of both organisms with little change in relative numbers of one type over the other until the low magnitudes of 10 or less per gram were reached.

Table 1, showing the combined ratios of *Bact. coli* to *Bact. aerogenes* in all of the stumps planted with feces, shows a sustained

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[1] The only planting made with animal feces.

characteristic fecal ratio of 100 or more *Bact. coli* to 1 *Bact. aerogenes*, irrespective of season, duration of the series, and magnitudes. Of the 22 items representing samples taken as many as 153 days after infection, only 4 show a reversal of the typical fecal relationship.
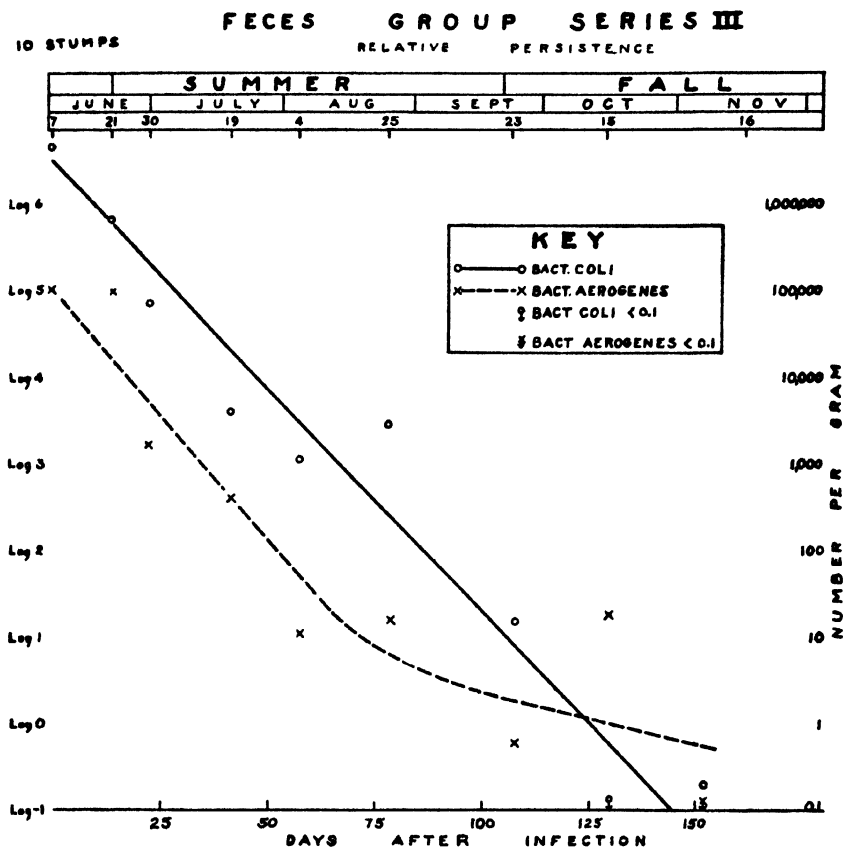
FIG. 1

The pure culture group

In series I of the pure culture group, ten stumps were planted December 27, 1929. The initial *Bact. coli* and *Bact. aerogenes* averages per gram were 323,000 and 708,000 respectively.

On the 6th day (December 2) two stumps showed an average of only 2.4 to 10.4 *Bact. aerogenes* per gram respectively. On the 19th day (December 15) these two and five of the others were negative. The remaining three in the series were first tested on the 22nd day (December 18). One showed an average count of 0.2 *Bact. coli* and 0.2 *Bact. aerogenes* per gram of material and

TABLE 1

Feces group. Consolidated data of series I, II and III—17 stumps

DATE	DAYS AFTER PLANTING	PERCENTAGE		RATIO OF BACT. COLI TO BACT. AEROGENES
		Bact. coli	Bact. aerogenes	
1930				
April 17..................	13	56 6	43.4	1.3: 1.0
June 21..................	14	87 2	12.8	67.6: 1.0
January 14..............	18	0	100.0	0 : 0 2
January 17..............	21	95.4	4 6	20.5: 1.0
June 30............	23	97.8	2.2	43.1: 1 0
January 21............. .	25	100.0	0	0.9: 0
April 30..................	26	100 0	0	5 4: 0
January 28..............	22	100.0	0	0.2: 0
May 14..................	40	0.08	99.92	1.0:1,250.0
July 19.................	42	88.4	11.6	76.4: 1 0
August 4......	58	98 6	1.4	63.1: 1:0
February 26..............	61	100.0	0	0 6: 0
June 4...................	61	62.7	37.3	15.0: 1.0
June 18..................	75	100.0	0	0.7: 0
August 25...............	79	98.9	1.1	89.1: 1.0
June 30.................	87	58.4	41.6	1.4: 1 0
September 23.............	108	92.7	7.2	12.6: 0
July 22..................	109	100.0	0	57.0: 0
July 29.................	116	0	100.0	0 : 112.0
October 15..............	130	100.0	0	0 : 12.5
November 6..............	152	100.0	0	0.2: 0
September 4.............	153	100 0	0	0.1: 0

the rest were negative. Samples taken on the 26th, 32nd, 41st, 63rd, 76th, 98th and 111th days were negative except one, which showed an average of 4.2 *Bact. aerogenes* per gram on the 76th day. Results were negative again on the 98th and 111th days.

The significant feature of this series is the failure of the infection to last as long as the first series of the feces group planted at

the same time, during the season of severe cold.   Moreover, only *Bact. aerogenes* was found in the few instances that were positive after the sixth day.   Their numbers, however, were very small.

Series II, comprising three stumps, is essentially the same as series I.   Plantings were made January 28 with an initial average content of 307,000 *Bact. coli* and 456,000 *Bact. aerogenes* per gram. Recoveries on the 9th, but not on the 29th day, showed 2.0 *Bact. coli* and 2.0 *Bact. aerogenes*, as an average per gram respectively. Samples on the 36th, 49th, 62nd and 77th days were negative.

Both series represent winter conditions.

Series III, of 8 stumps, was planted in the early spring (April 4), and the last recovery was made November 19 or the 229th day. More data were available in this series.

In figure 2 there is seen a falling off of numbers up to a time between the 41st and 61st days (May 15 and June 4).   From this time on, however, there appeared a gradual increase in the *Bact. aerogenes* content, reaching a peak on the 153rd day (September 4), after which there was a quick decline.   *Bact. coli* underwent a slight and irregular increase but in much smaller degree than *Bact. aerogenes*.   Thirteen of the 17 items in this series showed an excess of *Bact. aerogenes* over *Bact. coli*.   It should be recalled that in the corresponding series planted with feces, during this period *Bact. coli* maintained its typical fecal ratio to *Bact. aerogenes* throughout the experiment (over 100 to 1).

In series IV nine stumps, there is a similar picture (fig. 3). Plantings were made in the late spring (May 27) and the last recoveries of both *Bact. coli* and *Bact. aerogenes* were made on January 10, the 228th day.   There was a reduction in numbers of both organisms until the 75th day (August 11) when an increase of both began.   This reached a peak between the 148th and 170th days (October 22 and November 13) and declined again between the 183rd and 197th days (November 26 and December 10). Throughout the period the initial excess of *Bact. aerogenes* over *Bact. coli* was consistently maintained and was somewhat increased during the late summer and fall.   Only four of seventeen items showed a reversal of this relationship.

Table 2, representing the consolidated data of all the culture
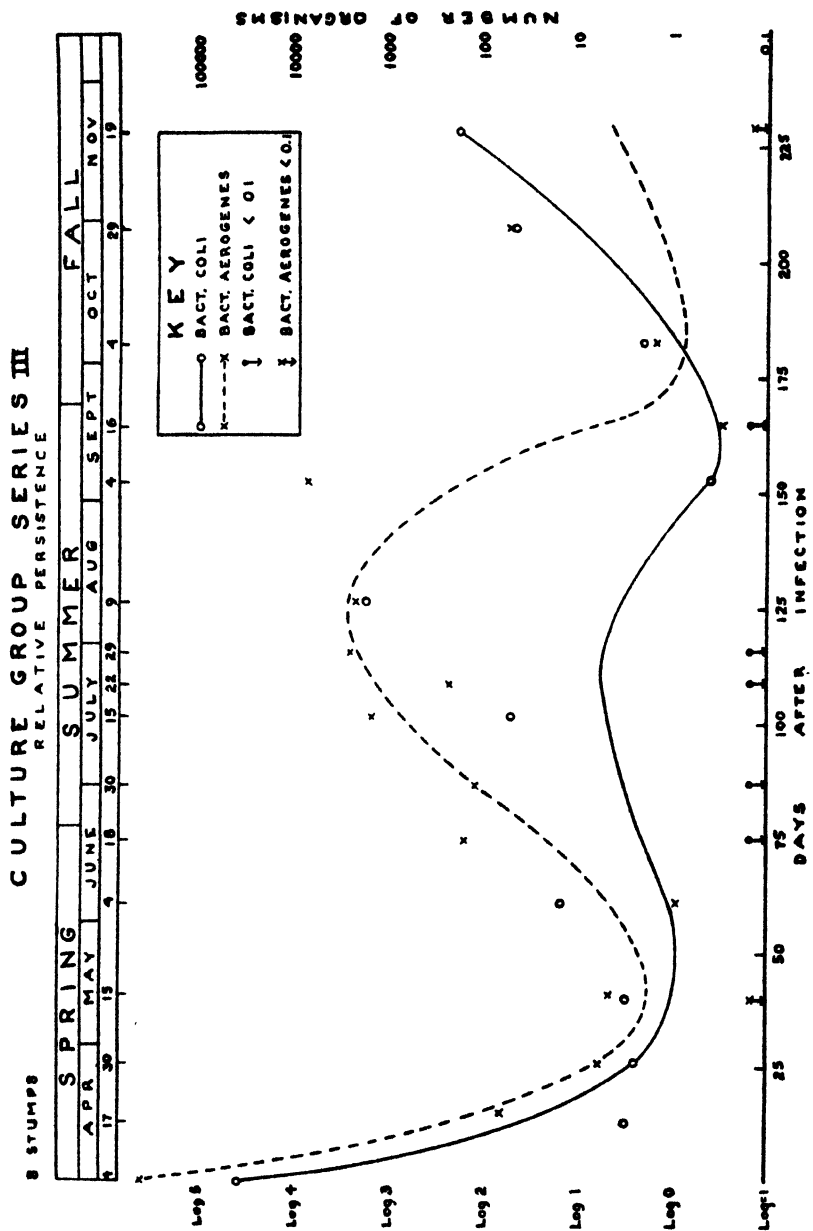
Fig. 2
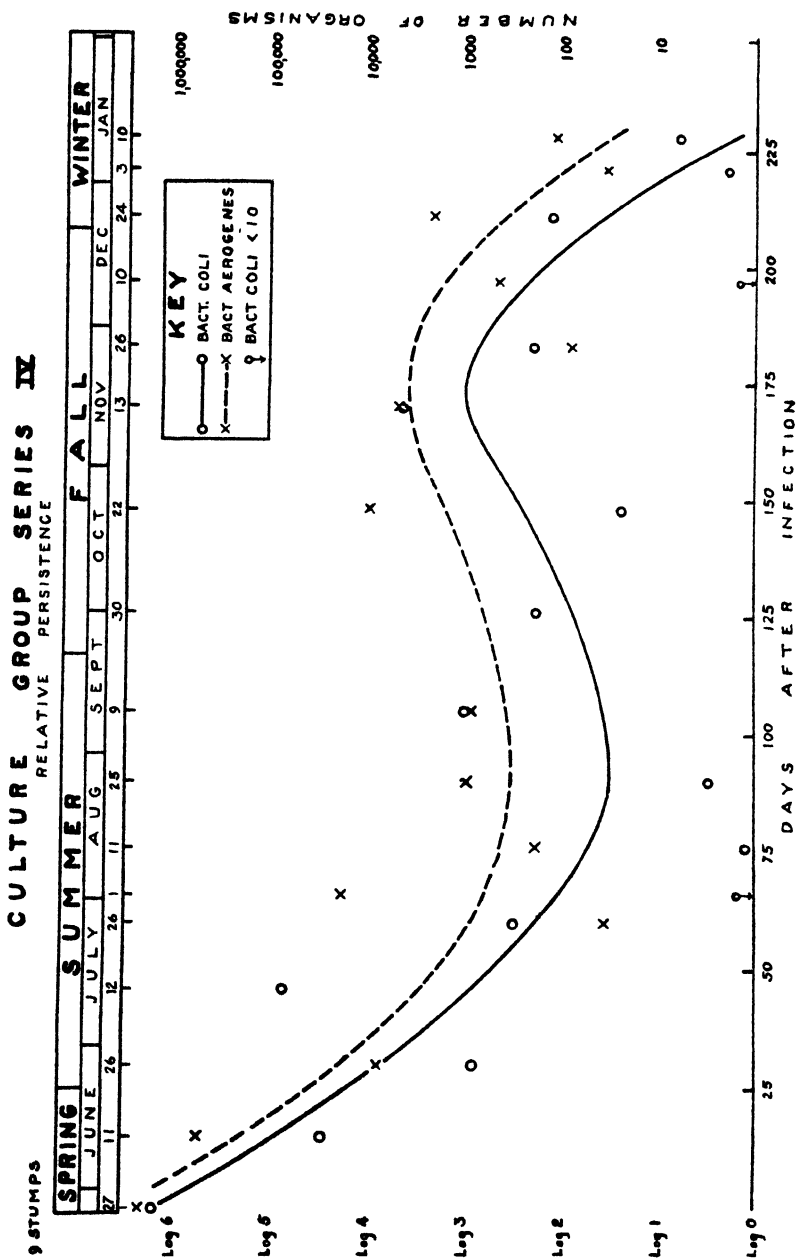
Fig. 3

plantings, shows a sustained ratio of *Bact. coli* and *Bact. aerogenes*, which is the converse of the typical fecal ratio, viz.: 1 or less

TABLE 2

Culture Group.   Consolidated data of series III and IV—17 stumps

| DATE | DAYS AFTER PLANTING | PERCENTAGE | | RATIO OF BACT. COLI TO BACT. AEROGENES |
		Bact. coli	Bact. aerogenes	
1930				
April 17	13	4.5	95.5	1.0: 20.6
June 11	15	50.7	49.3	1.0: 1.9
April 30	26	29.8	71.2	1.0: 2.4
June 26	30	9.2	90 8	1.0: 9.8
May 14	40	100.0	0	3.0: 0
May 15	41	0	100.0	0 : 4.6
July 12	46	88.8	11 2	29.8: 1.0
July 26	60	89.7	10 3	8.6: 1.0
June 14	61	94.4	5.6	16.9: 1.0
August 1	66	0	100.0	0 :18,300.0
June 18	75	0	100.0	0 : 158.0
August 11	76	0.6	99 4	1 0: 148.0
June 30	87	0	100.0	0 : 120.0
August 25	90	0 3	99 7	1.0: 322 0
July 15	102	3 3	96.7	1 0: 29.6
September 9	105	54.6	45.4	1.2: 1 0
July 22	109	0	100.0	0 : 225 0
July 29	116	0	100 0	0 : 2,580.0
September 30	126	52.7	47.3	1.1: 1.0
August 9	127	43.6	56.4	1.0: 1.3
October 22	148	0.2	99.8	1.0: 409.0
September 4	153	0 005	99 995	1.0:18,100.0
September 16	165	0	100.0	0 : 0.3
November 13	170	49.1	50.9	1 0: 1.0
October 4	183	58.3	41.7	1.4: 1.0
November 26	183	68.1	31.9	2.5: 1.0
December 10	197	0	100 0	0 : 472.0
October 29	208	45.8	54.2	1.0: 1.2
December 24	211	5.5	94.5	1.0: 17.1
1931				
January 3	221	14.5	85.5	1.0: 5.9
January 10	228	4.5	95.5	1.0: 21.0
November 19	229	100.0	0	180.0: 0

Bact. coli to 20 or more *Bact. aerogenes*, irrespective of season, duration of series, and magnitudes.   Of the 32 items representing

samples taken as many as 229 days after infection, only 7 showed a reversal of the relationship.

DISCUSSION

Relative rate of reduction

The sharpest percentage reduction of *Bact. coli* and *Bact. areogenes* occurred in the winter plantings of both feces and laboratory cultures. As the season advanced, however, the rate became slower up to the late spring plantings, in which it was slowest.

PERCENTAGE REDUCTION FROM INITIAL CONTENT
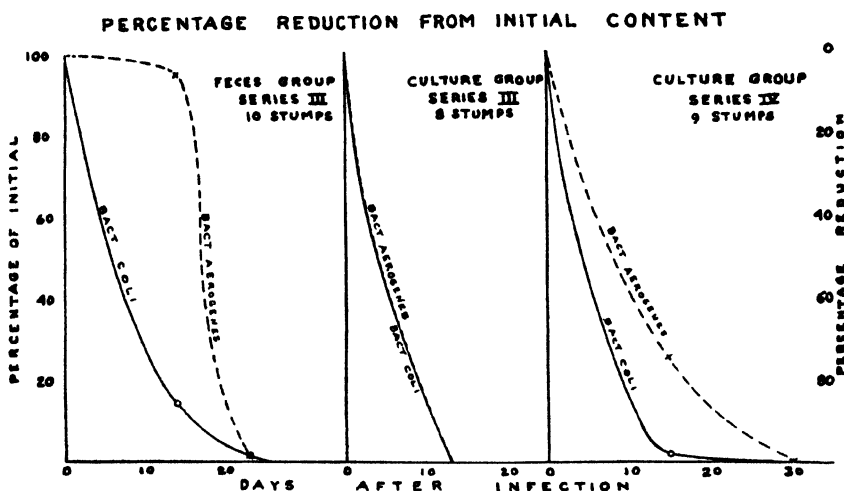
FIG. 4

There was very little difference in the relative rates of reduction throughout the experiments. Reduction occurred consistently but neither organism appeared to gain a significant ascendancy over the other during the period of decline. From 98 to 99 per cent reduction in numbers took place in 21 to 30 days. In figure 4 it is noted that the *Bact. coli* rate of decline in series III of the feces group and series IV of the culture group is somewhat faster than that of *Bact. aerogenes*. The rates of decline were practically the same for the two organisms in series III of the culture group.

Relative time of survival

It is seen that the time of survival of both organisms was much shorter in the winter plantings than in the late spring plantings. In the fecal group, *Bact. coli* and *Bact. aerogenes* lived 61 days and 21 days respectively in the winter plantings. In the culture group on the other hand, the corresponding relationships were: In winter, 9 days and 22 days respectively, and late spring, 228 days for both. It was obvious throughout the experiment that there was very little difference in the time of survival of the two organisms.

Relative rates of growth

In the case of winter plantings, no significant increase in the numbers of either type of organism occurred at any time in either the fecal or the pure culture group. In the spring and late spring plantings, however, growth of both types of organisms took place in the culture group of plantings. Only very slight growth occurred in the fecal group, appearing in series II of two stumps but not in series III of 10 stumps. In marked contrast to the feces plantings carried over the summer, the pure culture plantings (figure 2 of series III) exhibited a decided increase of *Bact. aerogenes* over *Bact. coli;* the latter increased only slightly in numbers. To a less degree, this was true in figure 3 of series IV. It is noted that the period of this increase is approximately 100 days, beginning about the 75th day and ending on or about the 170th day. The increase in each series began at different times but generally in the warmer months.

The points common to both organisms were: (1) that the growth occurred in the late summer and fall, (2) that the increases began about the 60th day after planting, reached a peak in 120 and 125 days and declined to the 60 day level by about the 170th day, (3) the phenomenon appeared to be independent of the initial magnitude of the numbers planted, and (4) the increased growth was largely confined to *Bact. aerogenes.*

The *Bact. coli* cultures underwent a slight corresponding increase, of which the peak in series IV lagged about 50 days behind that of *Bact. aerogenes.* The strains of *Bact. aerogenes* naturally

present in the feces plantings, did not at any time outgrow the *Bact. coli* in the same series.

<center>SUMMARY AND CONCLUSIONS</center>

In these experiments:

1. Under winter conditions, *Bact. coli* and *Bact. aerogenes*, both from fecal material and from cultures, underwent rapid decline, without exhibiting any significant change in relative numbers.

2. At no time was there a material difference in the surviving time of either type of organism in feces.

3. In one of the pure culture series, there was a marked increase in *Bact. aerogenes* beginning about 60 days after infection, accompanied by only a slight increase in *Bact. coli*. The peak of growth was reached about the 125th day and there was subsequent decline to about the 60-day level by the 170th day. Thereafter, relative numbers continued about equal up to the time when both organisms disappeared.

4. The rather marked relative increase in growth of *Bact. aerogenes* over *Bact. coli* which occurred in certain culture plantings suggests the possibility that the commonly observed predominance of *Bact. aerogenes* in nature may be due in part to greater multiplication of certain hardy strains of *Bact. aerogenes*.

5. Under such circumstances, the *Bact. aerogenes* count, if accepted as a quantitative index of fecal pollution, would tend to distort the sanitary picture, yielding no evidence of the recency of the contamination, and giving an exaggerated idea of the amount of pollution after two months, at which time the pollution is less dangerous from the standpoint of water-borne disease than at the outset.

6. The *Bact. coli* count, on the other hand, had a more consistent relation to the initial amount of fecal pollution, and to the time elapsing after its occurrence, both of which factors are of essential importance in judging dangerous pollution.

7. The high resistance of the colon-aerogenes group of organisms to the action of the elements was evident in the tests, and gives further assurance that as test organisms, their survival-time

affords a safe margin over that of the less resistant intestinal pathogens which constitute the real danger from fecal pollution.

8. The plate count method in ferrocyanide-citrate agar was found to be a sensitive measure of changes in numbers of colon-aerogenes organisms occurring under the conditions of this experiment.

9. The fact that *Bact. coli* and *Bact. aerogenes* will live a long time in wood under natural conditions, has a practical bearing on the quality of water from any supply in which the water is in contact with wood. Often wooden storage tanks are used, especially in small supply systems. In older types of private wells, wooden casing is commonly used, and sometimes wooden troughs or conduits as well. There also still remain a few "old oaken buckets." Under such conditions the presence of *Bact. aerogenes* alone is probably of no sanitary significance.

REFERENCES

CHEN CHONG CHEN AND RETTGER, L. F. 1920 A correlation study of the colon-aerogenes group of bacteria with special reference to the organisms occurring in soil. Jour. Bact., **5**, 253.

HINMAN, JACK, J., JR. 1925 Importance of differentiating colon-aerogenes group in examining water. Amer. Jour. Pub. Health, **15**, 614.

KOSER, S. A. 1927 The sanitary significance of differential tests for the coli-aerogenes group. Amer. Jour. Pub. Health, **17**, 1178.

TONNEY, FRED O., AND NOBLE, RALPH E. 1930 The relation of direct *Bact. coli* and *Bact. aerogenes* counts to sources of pollution. Jour. Amer. Water Works Assoc., **22**, 488–501.

TONNEY, FRED O., AND NOBLE, RALPH E. 1931 An improved ferrocyanide-citrate agar for direct plating of colon-aerogenes organisms. Jour. Amer. Water Works Assoc., **23**, 1202.

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